INVESTIGATIONS on the PRODUCTION of LONG CHAIN FATTY ACIDS in CHOANEPHORA CUCURBITARUM (Berk. & Rav.) Thaxter

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ABSTRACT

The fatty acid composition of the total cellular lipids of Choanephora cucurbitarum incubated for 96 hrs on either glucose-ammonium sulfate or malt-yeast extract media was determined. The major fatty acids were palmitic, palmitoleic, stearic and linoleic acids. The saturated fatty acid possessing the longest acyl chain was stearate (C 18:0). The presence of glutamic acid (2.0×10^{-1}% or 1.36×10^{-2}M) in either of the above growth media resulted in increase in percent of γ-linolenic acid, decrease in percent of linoleic acid and appearance of a new series of fatty acid $> C_{18}$ e.g. $C_{22:0}$, $C_{24:0}$, $C_{26:0}$. The addition of glutamic acid had no effect on the lipid yield but slightly decreased the degree of unsaturation.

Compounds which duplicated the effect of glutamic acid were acetate, malate, citrate, succinate, α-ketoglutarate, proline, γ-aminobutyric acid and glucose (3%) but not aspartic acid or alanine.

No correlation was found between glutamic acid pool concentration and the presence in the growth medium of those compounds which stimulate long chain fatty acid production.

Four hours of incubation with 27 μM glutamate supported the production of long chain fatty acids. This stimulation is inhibited if 272 μM isophthalic acid is added with 27 μM glutamate. But, long chain fatty acids were detected when 27 μM α-ketoglutarate is also present in the incubation mixture.

Five hours of incubation with 100 μg/ml of cycloheximide resulted in over 90% inhibition of cytoplasmic protein synthesis. Glutamate (27 μM) enhanced the synthesis of long chain fatty acids under these conditions.

These findings are discussed in an attempt to provide a plausible explanation common to compounds that support the production of long chain fatty acids.

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INTRODUCTION

The fatty acid composition of most micro-organisms is influenced by the environment in which they are grown. Parameters such as age, carbon source, temperature and O_2 content affect the degree of unsaturation and the chain length of fatty acids (Jollow et al, 1968; Erwin, 1973; Weete, 1974). The exact manner whereby these changes come about at molecular level is not fully understood. Nevertheless, the fact that an organism grown at various temperatures adjusts its degree of unsaturation can be appreciated since membrane fluidity is essential for the proper functioning of membrane bound proteins (Johnson and Davenport, 1971).

Deven and Manocha (1975) reported that glutamic acid influences the fatty acid composition of Choanephora cucurbitarum. When this fungus is grown on malt-yeast extract or glucose-ammonium nitrate media, the fatty acid possessing the longest hydrocarbon residue synthesized is γ-linolenic acid (γ 18:3, cis-6, cis-9, cis-12, octatrienoic acid). But, when glutamic acid is added to either of the above media a number of fatty acids beyond γ-linolenic acid ($C_{\gamma\ 18:3}$) were detected. The fatty acids, e.g. $C_{22:0}$, $C_{22:1}$, $C_{24:0}$, and $C_{26:0}$ hitherto referred to as long chain fatty acids, were never observed in the absence of glutamic acid.

This was probably the first report showing that addition of an amino acid (glutamic acid) can result in the synthesis of long chain fatty acids. Thus the question to be asked is; "How does the presence of glutamic acid support the production of detectable amounts of long chain fatty acids?".

The long chain fatty acids are synthesized from existing fatty acids by microsomal or mitochondrial elongating enzymes (Hitchcock and Nichols, 1971; Weete, 1974). The elongating reactions catalyzed by mitochondrial (I) and microsomal (II) pathways may be illustrated as the following:

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I $RCOSCoA + CH_3COSCoA \xrightarrow{NAD(P)H} RCH_2CH_2COSCoA$

II $RCOSCoA + HO_2CCH_2COSCoA \xrightarrow{NAD(P)H} RCH_2CH_2COSCoA$

The present investigation is an attempt to clarify the circumstances under which glutamic acid stimulates the production of long chain fatty acids. Various possible mechanisms of glutamic acid action predicted by equation I and II are listed below.

Glutamic acid may:

1) increase substrate concentration i.e. acetyl-CoA, malonyl-CoA, reduced nucleotide, or acyl-CoA
2) facilitate product removal e.g. acyl-CoA
3) induce the synthesis of new proteins(s)
4) increase enzyme activity by providing a positive effector
5) increase enzyme activity by removing a negative effector

The actual mechanism may be further complicated by simultaneous occurrence of more than one of these possibilities. Experiments using gas-liquid chromatography, amino acid analysis and various metabolic inhibitors have been carried out. The findings have been examined in an attempt to find plausible explanation for the effect of glutamic acid on the fatty acid composition of Choanephora cucurbitarum.

LITERATURE REVIEW

When biological material is extracted with one or more low polarity solvents such as ethanol, ether, chloroform, benezene, petroleum ether etc., a portion of the material may dissolve. The name "lipid" is given to the components of this soluble fraction (Weete, 1974).

Lipids are divided into two groups, complex and simple lipids, on the basis of their chemical reactivity in the presence of an alkali (Lehninger, 1975). The characteristic feature of complex lipids is that they contain fatty acids which are covalently linked to various head groups, e.g. glycerol phosphate, sphingosine or a carbohydrate moiety. Simple lipids generally do not contain fatty acids and they include substances such as terpenes, steroids and prostaglandins.

Fatty acids are monocarboxylic acids with hydrocarbon residues that may by straight chain, branched, saturated or may contain one or more double bonds (Weete, 1974). In fungi, the length of the hydrocarbon residues ranges from a chain length of C_{10} to C_{26}, but the major fungal fatty acids possess hydrocarbon chains of 16 and 18 carbon atoms. The purpose of this review is to consider in detail the formation of fatty acids and the factors which affect their chain length.

I De Novo Synthesis of Fatty Acids

The metabolism of fatty acids was studied earlier than any other class of lipids (Johnson and Davenport 1971; Weete, 1974). As early as 1904 F. Knoop observed that, when dogs were fed an even carbon ω -phenylalkanoic acid, phenylacetic acid was excreted in the urine, regardless of the length of carbon chain of alkanoic acid. On the other hand, when odd carbon ω-phenylalkanoic acids were fed, benzoic acid was excreted. From these experiments, Knoop concluded that fatty acids are degraded by oxidative

removal of successive two carbon fragments starting from the carboxyl end and thus postulated the β-oxidation theory of fatty acid catabolism. It was some fifty years later that the process and the individual steps involved in β-oxidation were verified (Lehninger, 1975).

Investigations leading to the elucidation of fatty acid biosynthesis lagged behind studies of degradation. At one time it was widely accepted that fatty acids are synthesized by reversal of β-oxidation pathway of fatty acid catabolism (Lynen, 1961). However, observations such as the inability of purified β-oxidation enzymes to synthesize fatty acids longer than four carbon atoms and the requirement of bicarbonate for fatty acid synthesis led to the conclusion that a different enzyme system is involved in the de novo synthesis of fatty acids (Wakil, 1961; Hitchcock and Nichols 1971).

Much of the pioneering work leading to the elucidation of the mechanism of de novo synthesis was performed using pigeon liver (Wakil, 1961), yeasts (Lynen, 1961) and E.coli (Vagelos et al 1966). Later fatty acid synthesizing systems capable of carrying out de novo synthesis were also characterized in plant tissues (Heinstein and Stumpf, 1969).

There are two enzyme systems involved in the de novo synthesis; acetyl-CoA-carboxylase and fatty acid synthetase (Volpe and Vagelos, 1973). The overall reaction of these two enzyme systems is given as follows (Weete, 1974):

$$\text{Acetyl-CoA} + \text{Malonyl-CoA} + 2n\text{NADPH} + 2n\text{H}^{+} \rightarrow \text{CH}_3(\text{CH}_2\text{-CH}_2)_n\text{-CO-CoA} + n\text{CO}_2 + 2n\text{NADP}^{+} + 2n\text{H}_2\text{O}$$

The "primer" for fatty acid synthesis is acetyl-CoA and the chain is built up by successive condensation with malonyl-CoA. Malonyl-CoA is synthesized from acetyl-CoA and bicarbonate. This reaction is the first committed

step in the de novo fatty acid synthesis which may be illustrated as follows:

$$ATP + HCO_3^- + CH_3CO\text{-}CoA \xrightarrow[\text{carboxylase}]{Me^{++}\ \text{acetyl-CoA}} O_2CCH_2COSCoA + ADP + Pi$$

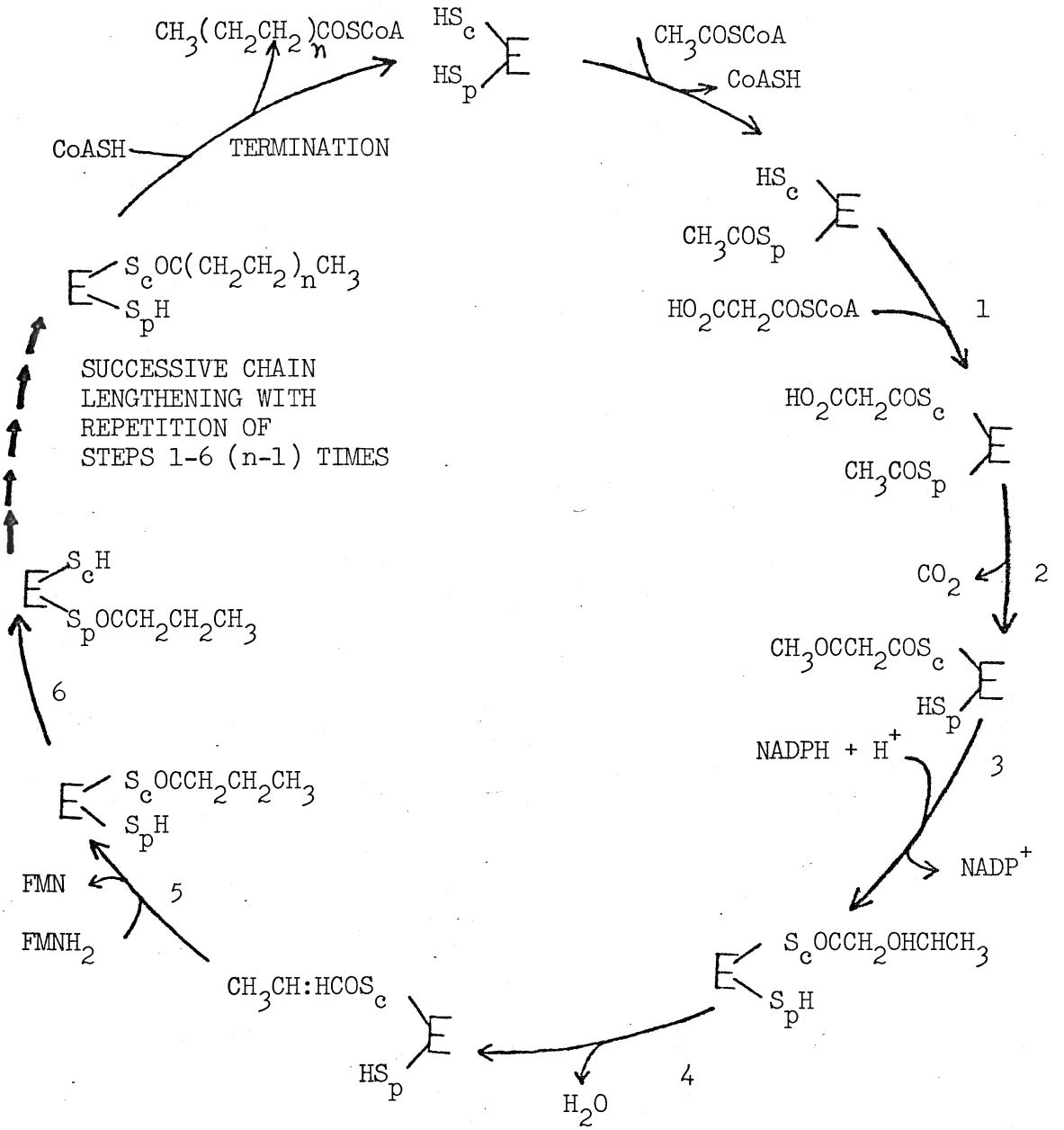
The remaining reactions are catalyzed on the multienzyme fatty acid synthetase complex. They are illustrated in Fig. 1. The details of the individual steps involved in the fatty acid synthesis are described in a number of research papers (Johnson and Davenport, 1971; Hitchcock and Nichols, 1971, Vagelos, 1974; Weete, 1974 and Volpe and Vagelos, 1976).

The major pathway for de novo fatty acid synthesis occurs in the cytoplasm. Yet, acetyl-CoA the substrate for fatty acid synthesis is synthesized mainly in the mitochondrion. Since acetyl-CoA cannot permeate the mitochondrial membrane, it must be modified in some way so that C_2-units can be transferred through the membrane and may become available to the fatty acid synthesizing complex in the cytoplasm. This may be accomplished in several ways (Weete, 1974; Gurr and James, 1975):

1) acetyl-CoA may be broken down to acetate which can freely permeate the membrane.
2) acetyl-CoA may be converted to citrate and then transported across the membrane.
3) acetyl-CoA may be shuttled to glutamate which can permeate the mitochondrial membrane.
4) the acetyl part may be transferred to carnitine and the resulting product may be transported through the membrane.

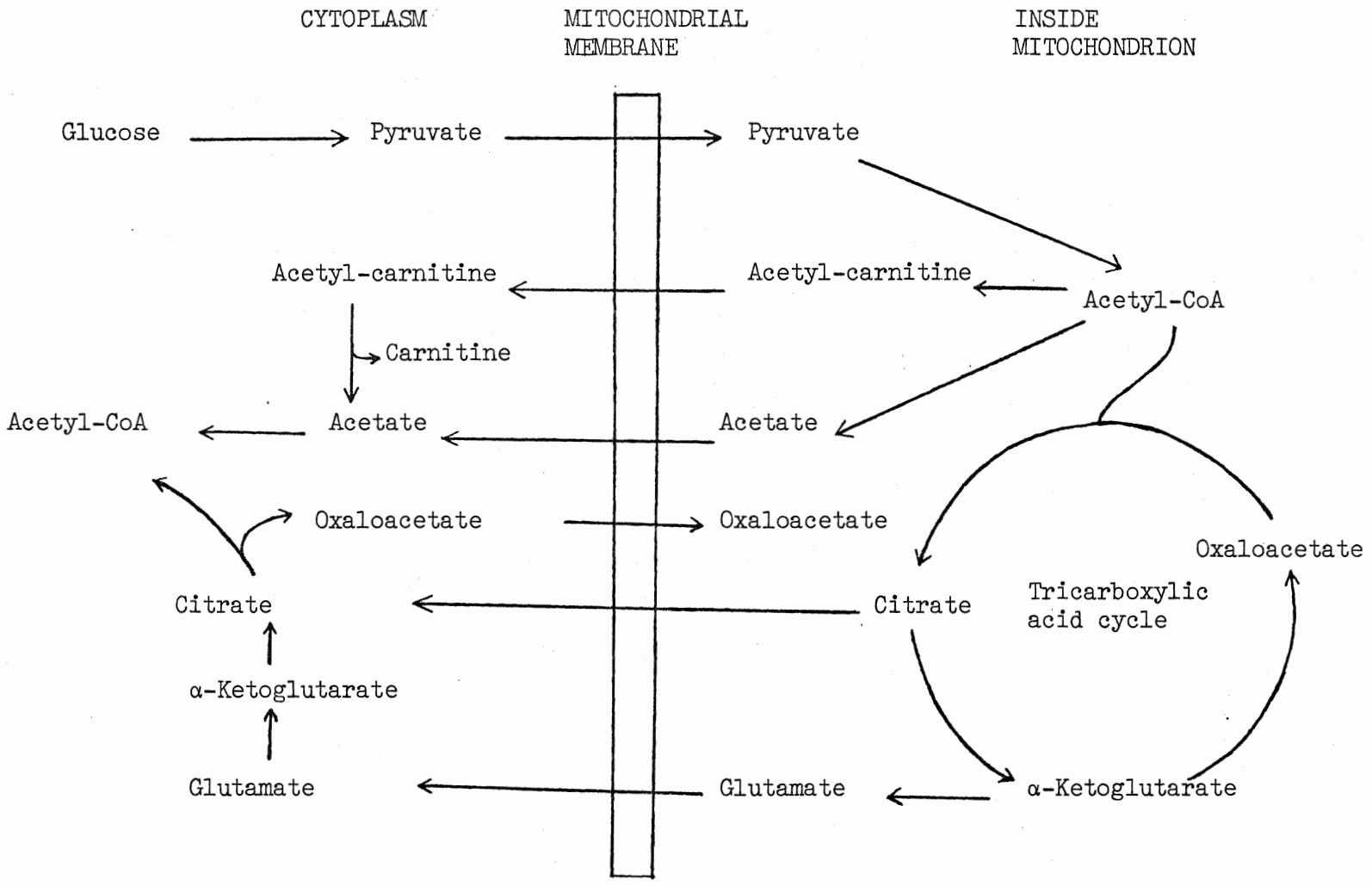
These processes are illustrated in Fig. 2. Once the C_2 unit is transported into the cytoplasm, acetyl-CoA can be regenerated.

Fig. 1 General scheme of fatty acid biosynthesis in yeast, Lynen (1967)



The fatty acid synthetase is denoted by $\begin{matrix} HS_c \\ HS_p \end{matrix}$E. The subscripts "c" and "p" refer to central and peripheral ACP(Acyl Carrying Protein) thiol binding sites on the enzyme complex respectively.

Fig. 2 Generation of extramitochondrial Acetyl-CoA, Gurr (1975)



The principal product of mammalian fatty acid synthetase is palmitic acid ($C_{16:0}$) whereas yeast enzyme produces mainly palmitic and stearic ($C_{18:0}$) acids as the predominant fatty acids. Yet, the fatty acid synthetases from both of these sources synthesize fatty acids with carbon chain lengths less than C_{16}. One question to be asked: "Why are the fatty acids $C_{16:0}$ and $C_{18:0}$ the predominant products of fatty acid synthetase?" Before answering this question, we need to consider the details of termination reactions.

A. Termination Reaction

The substrates and the intermediates of fatty acids are covalently bound to the fatty acid synthetase complex via a thioester linkage. In the case of yeast, these intermediates are not released until the products palmitate and stearate are formed. The enzyme-bound intermediate is acyl-ACP. The acyl chain may be removed as the ACP ester, CoA ester or as a free acid (Weete, 1974; Hitchcock and Nichols, 1971). In the case of yeast the acyl chain is transferred to CoA; hence the final product is acyl CoA. On the other hand, mammalian fatty acid synthetase often transfers acyl chains to water molecules hence the resultant is a free acid.

B. Control of Termination Reaction

There are two reactions open to newly formed enzyme bound acyl chain. It can be transferred either to CoA (termination reaction) or to "peripheral" thiol group so that incoming malonyl-CoA can continue the chain lengthening process.

Sumper et al (1969) proposed that the probability of any covalently acyl residue forming a product by being transferred to CoA is determined by relative rates of elongating and terminating transfer reaction of the synthetase, and the growing alkane chain interacts with the enzyme protein only after 13 carbon atoms have been attained. This interaction changes the

relative velocities in favour of product formation by an energy increment of -0.9 Kcal for each additional methylene group beyond the 13th carbon. Schwiezer et al (1970) pointed out that the "central" thiol group is spatially separated from the active centre of the transferase until the acyl chain lengths of C_{16} and C_{18} are reached. With chain elongation the acyl group becomes increasingly lipophilic which may cause conformational changes in the quaternary structure of the fatty acid synthetase. Thus an acyl chain becomes available for the transferase reaction. This mechanism of chain termination could conceivably operate in mammalian and other tissues.

II Factors Influencing the Chain Length of de novo Synthesized Saturated Fatty Acids

A. Substrate Concentration

It is known that the concentration of malonyl CoA affects the pattern of fatty acids produced both in animal (Smith and Dils, 1966; Bartley et al, 1967) and in plant (Yang and Stumpf, 1965) systems. Both plant and animal fatty acid synthetases synthesize short chain (i.e. $<C_{14:0}$) acids when they are incubated in the presence of limiting amounts of malonyl-CoA. Smith and Dils, (1966) reported that the purified lactating mammary gland fatty acid synthetase produces predominantly C_{14} and C_{16} acids in the presence of 500 μM malonyl-CoA. But, when malonyl-CoA concentration is increased to 2500 μ M, stearic ($C_{18:0}$) acid is detected which was never detected under any other experimental conditions.

The effect of low malonyl-CoA concentration on the pattern of fatty acids produced may be explained on the basis of a model proposed by Sumper et al (1969). If the rate of malonyl-CoA condensation to fatty acid synthetase is decreased, then the rate of elongation is also decreased. Thus the acyl chain spends more time on the enzyme complex than usual and hence

could become available for transferase reaction prior to reaching the normal length of C_{16} or C_{18}.

B. Thiolester Transferase

Lactating rabbit mammary glands synthesize octanoic ($C_{8:0}$) and decanoic ($C_{10:0}$) acids both in vivo and in vitro. But, the highly purified fatty acid *(Knudsen and Dils, 1975). When particle free supernatant was added back to purified synthetase, the predominant fatty acids were octanoic and decanoic acids. It was postulated that particle free supernatant fraction contained a factor which alters the chain length of the fatty acids synthesized by the purified enzyme complex from $C_{14:0}$ - $C_{16:0}$ to $C_{8:0}$ - $C_{10:0}$. This factor was identified as an acyl thiol ester transferase (Hydrolase) with M.W. of 29,000 Daltons (Knudsen et al, 1976). The authors therefore claim that the occurrence of $C_{8:0}$ - $C_{10:0}$ fatty acids in lactating rabbit mammary gland is due to the presence of an acyl thiol hydrolase which specifically releases acids of short chain lengths from the acyl carrier protein of the fatty acid synthetase complex.

C. Oxygen Tension

The fatty acid profile of yeast grown aerobically shows palmitoleic acid $C_{16:1}$ and oleic acid $C_{18:1}$ as the major fatty acids. But when it is incubated anaerobically, the major fatty acids produced are of chain lengths $C_{10:0}$, $C_{12:0}$ and $C_{14:0}$ (Jollow et al, 1968). Further analysis of lipids from anaerobically grown yeast showed that the latter fatty acids are incorporated into phospholipids at the positions occupied by unsaturated fatty acids under aerobic conditions (Johnson and Davenport, 1971).

Interfacial properties of phospholipids are critically dependent on the structure of fatty acids (Meyer and Bloch, 1963). Introduction of a

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synthetase synthesizes predominantly C14:0 and C16:0 fatty acids

short chain ($C_{10:0}$, $C_{12:0}$) saturated fatty acid into phospholipids in place of unsaturated fatty acid (oleic acid) alters the physical properties of these lipids. Thus, the enhanced synthesis of C_{10} and C_{12} saturated fatty acids by anaerobic yeast may be an attempt by the organism to produce a phospholipid having physichochemical properties similar to phospholipids synthesized aerobically.

The question of interest is "How is the mechanism of chain length termination altered in the absence of oxygen, so as to produce $C_{10:0}$ and $C_{12:0}$ acids as the major species instead of $C_{16:1}$ and $C_{18:1}$ acids?" This question has received very little attention and the mechanism is not known. However, it may be postulated that in the absence of oxygen:

1. the intracellular malonyl-CoA concentration decreases
2. a new acyl transferase specific for $C_{10:0}$ and $C_{12:0}$ is synthesized
3. a new fatty acid synthetase is synthesized specific for short chain fatty acids.

The mechanism by which chain elongation is terminated during de novo fatty acid synthesis is not fully understood. It is probably not due to any single factor but to a combination of factors which are influenced by the dynamic balance of intermediary metabolism.

III Fatty Acid Elongation

Yeast fatty acid synthetase under certain conditions can synthesize $<C_{14}$ fatty acids as major components instead of C_{16} and C_{18} acids but under no conditions yet known does de novo fatty acid synthesis proceed above C_{18} (Weete, 1974). However, fatty acids with chain length $> C_{18}$ also exist. These fatty acids are synthesized by elongation of existing fatty acids.

There are two distinct elongation pathways present in the cell (Harlan and Wakil, 1963; Nugteren, 1965; and Guchhait, et al, 1966). They may be

distinguished on the basis of their specificity for the C_2 unit and their location in the cell. Elongating enzymes requiring malonyl-CoA are associated with the endoplasmic reticulum and those utilizing acetyl-CoA are associated with the mitochondrion (Hitchcock and Nichols, 1971).

A. The Acetyl-CoA Dependent Elongation

The overall reaction scheme of mitochondrial elongation may be expressed as the following:

$$R\text{-}COS\text{-}CoA + CH_3COSCoA + 2NAD(P)H + 2H^+ \xrightarrow[\text{enzymes}]{\text{elongating}} RCH_2CH_2COSCoA + 2NAD(P)^+ + CoASH + H_2O$$

The C_2 unit in mitochondrial elongation is acetyl-CoA. The "primer" molecule is acyl-CoA. The best "primer" of mitochondrial enongation is $C_{10:0}$ or $C_{12:0}$ acyl-CoA (Hinsch and Schubert, 1975; Bond and Pynodath, 1976). Unsaturated CoA derivatives can also act as "primers", though poorly, especially those with two or more double bonds.

i. Location in the Mitochondrion

Mitochondria have enzymes for both de novo and chain elongating biosynthetic pathways (Harlan and Wakil, 1963; Barron, 1966; and Howard, 1968). The major site of chain elongation is the outer membrane whereas, the inner membrane is responsible for de novo synthesis (Dahlen and Porter, 1968; Whereat et al, 1969; and Bond and Pynodath, 1976). Using rabbit heart mitochondria, Whereat et al (1969) reported that the outer membrane synthesized fatty acids of chain length C_{14}- C_{24} by addition of 1 or 2 acetate units, whereas the inner membrane had C_{14}- C_{18} acids as the major product, the result of de novo synthesis. Bond et al, (1976) characterized both synthetic systems of beef liver mitochondria. The outer membrane synthetic complex had M.W. of 126,000 Daltons and the inner membrane complex was 57,000 Daltons. When the outer membrane complex was incubated with myristoyl-CoA

and $\left[1 - {}^{14}C\right]$ Acetyl-CoA, the products were 42% palmitate ($C_{16:0}$), 24% stearate ($C_{18:0}$) and 22% arachidate ($C_{20:0}$) and longer chain fatty acids. A similar experiment with the inner membrane complex yielded mainly palmitic acid.

ii. Cofactor Requirement

The cofactors required for mitochondrial elongation are ATP, CoA, Mg^{++} and both NADH and NADPH.

Although ATP is required for the elongation reaction, it could be omitted if the "primer" is fatty acyl CoA instead of a fatty acid (Harlan and Wakil, 1963; Dahlen and Porter, 1968). Hence the proper substrate for chain elongation is an acyl-CoA not a fatty acid.

Both CoA and Mg^{++} are required for the synthesis of long chain fatty acids (Whereat, 1971). Mitochondrial requirement for CoA and Mg^{++} is dependent upon the manner of preparation of the mitochondria. Carefully prepared intact mitochondria have sufficient CoA and Mg^{++} for optimal rates of synthesis. The requirement of addition of these cofactors for optimal rate of sythesis implies some damage to membranes has occurred during preparation (Whereat, et al, 1969).

The nucleotide specificity for mitochondrial chain elongation is dependent on the tissue type. Hinsch et al (1976) reported that, liver, kidney cortex and brown adipose tissue mitochondria need both NADH and NADPH whereas heart muscle and aortic mitochondria require only NADH for optimal activity. Shimakata et al (1977) working with Mycobacterium smegmatis demonstrated that avidin-insensitive elongation required NADH as the sole hydrogen donor.

iii. Control of Acetyl-CoA Dependent Elongation

Rabbit heart mitochondria synthesize both short chain ($C_{12} - C_{16}$) and long chain fatty acids. When these mitochondria are incubated in the

presence of Krebs cycle intermediates, there is stimulation in the synthesis of both short chain and long chain fatty acids and a simultaneous large reduction of NAD (Whereat et al, 1967). These two observations were the germ seed for the present knowledge of regulation of heart mitochondrial fatty acid elongation.

Whereat (1971) hypothesized that Krebs cycle acids increased the intramitochondrial NADH:NAD^+ ratio. As a result there is reversed electron flow, i.e. NADH to fatty acids instead of fatty acids to NADH. Thus postulated, the mechanism of mitochondrial elongation is the reversal of β-oxidation. Furthermore, this elongation reaction is regulated by intramitochondrial NADH/NAD^+ ratio. Whereat also realized that this cannot be the universal mechanism of mitochondrial elongation since succinate has no effect on fatty acid elongation of mitochondrial preparations from liver and kidney (Whereat, 1971).

The detail mechanism of control exerting factors involves the knowledge of reaction sequence and the rate limiting step. The reaction sequence is given as follows (Barron and Mooney, 1970: and Seubert and Podack, 1973).

$$RCOSCoA + CH_3COSCoA \xrightarrow{\text{thiolase}} RCOCH_2COSCoA + HSCoA$$

$$RCOCH_2COSCoA + NADH + H^+ \xrightarrow[\text{dehydrogenase}]{\beta \text{ hydroxy acyl CoA}} RCHOHCH_2COSCoA$$

$$RCHOHCH_2COSCoA \xrightarrow{\text{enoyl CoA hydratase}} RCH{=}CHCOSCoA + H_2O$$

$$RCH{=}CHCOSCoA + NAD(P)H + H^+ \xrightarrow[\text{reductase}]{\text{enoyl-CoA}} RCH_2CH_2COSCoA + NAD(P)^+$$

The malonyl-CoA independent fatty acid chain elongation occurs with reversal of three steps in β-oxidation (Seubert and Podack, 1973). In the last step of synthetic process a new enzyme, enoyl-CoA reductase, is used for the reduction of unsaturated acyl CoA. This step has free energy of -14 Kcal/mole, hence the synthetic reaction can occur. The rate limiting reaction is the last step catalyzed by enoyl-CoA reductase. There are two

types of enoyl reductase, the heart type which is NADH linked and the liver type which is NADPH linked (Seubert and Podack, 1973: Hinsch et al, 1976). The rate limiting factors are the level of intramitochondrial NADH and NADPH. The substrates affecting NADH level are succinate and other Krebs cycle acids, whereas those affecting the level of NADPH are citrate, isocitrate, glutamate, α-ketoglutarate, malate, fumarate but not succinate (Seubert and Podack, 1973).

Hinsch et al (1976) proposed different roles of mitochondrial chain elongation on the basis of nucleotide requirement. The chain elongating system, requiring only NADH, may be of importance in the conservation of reducing equivalents or acetate units in the anaerobic state or high phosphate potential and those requiring both NADH nad NADPH may play a role in the transfer of hydrogen from NADPH to the respiratory chain.

B. Malonyl-CoA Dependent Elongation

The information about microsomal elongation systems is far scarcer than the mitochondrial chain elongation system. Microsomal chain elongation is associated with the endoplasmic reticulum (Nugteren, 1965; and Guchhait et al, 1966). The elongation process occurs by successive addition of malonyl-CoA to acyl-CoA "primer".

$$\begin{array}{l} \qquad\qquad\quad \text{COOH} \\ \text{R-COSCoA} + \underset{\downarrow}{\text{CH}_2}\text{-COSCoA} + \text{NAD(P)H} + \text{H}^+ \\ \qquad\quad \text{CO}_2 \swarrow \text{microsomal elongating system} \\ \qquad \text{R-CH}_2\text{-CH}_2\text{SCoA} \end{array}$$

The synthetic mechanism is supposedly similar to that of cytoplasmic de novo synthesis (Podack et al, 1974). There is some evidence to indicate that there are different types of elongating enzymes within the microsomes (Goldberg et al, 1973; Bloch and Vance, 1977). Goldberg et al, (1973) working with quaking mice, deduced the existence of these elongating enzymes which

synthesize stearate ($C_{18:0}$), arachidate ($C_{20:0}$), and behenate ($C_{22:0}$) and lignocerate ($C_{24:0}$) from palmitate with the addition of one or two malonyl-CoA units.

The unsaturated acyl-CoA "primers" show the optimal activity for elongation (Nugteren, 1965). Thus it appears that the unsaturated fatty acids are the true substrates of microsomal elongating enzymes. The biosynthesis of arachidonic acid ($C_{20:4}$) from its precursor γ-linolenic acid ($\gamma C_{18:3}$) necessitates elongation prior to desaturation and not vice versa. Hence, microsomal elongation could function to elongate the unsaturaged fatty acids so that further desaturation may take place (Seubert and Podack, 1973).

IV Aerobic Desaturation of Fatty Acids

In general, during aerobic desaturation, unsaturated fatty acids are synthesized from more saturated precursors by oxidative removal of two hydrogen atoms (Gurr, 1974):

$$R\text{-}CH_2CH_2(CH_2)yCOSX + O_2 + NAD(P)H + H^+ \xrightarrow{\text{desaturase}} RCH = CH(CH_2)yCOSX + NAD(P) + 2H_2O$$

The introduction of the first double bond in plants, animals, yeasts and bacteria occurs at carbon atoms 9 and 10 counting from the carboxyl end. The position of the second and subsequent double bonds occur with a methylene interrupted pattern. In plants, the second and subsequent double bonds are introduced between the first double bond and the terminal methyl group, whereas in animals, the double bonds are introduced toward the carboxyl group. Some organisms like Euglena are able to desaturate in both directions (Hitchcock and Nichols, 1971).

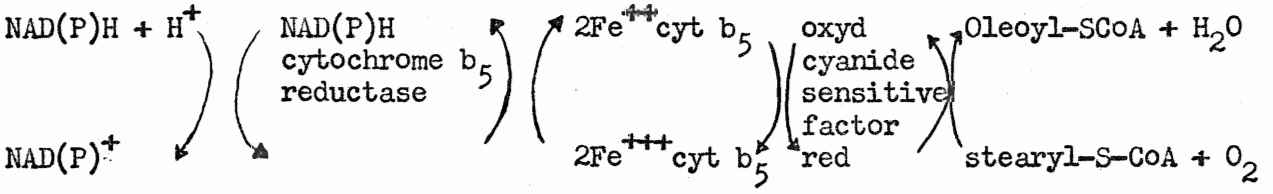
As a result of the different manners of desaturation by various organisms, distinct families of polyunsaturated fatty acids occur (Weete, 1974). Members of each family can easily be recognized by numbering the fatty acids from

the terminal methyl group. The three important families of polyunsaturated acids are $\omega 3$, $\omega 6$ and $\omega 9$. The symbol "ω" designated that numbering started from the methyl terminal carbon atom. Thus $\omega 9$ indicates that the first double bond is at the 9th carbon atom. Oleic, linoleic and α-linolenic acids are examples of $\omega 9$, $\omega 6$ and $\omega 3$ families respectively.

The desaturating enzymes are associated with the microsomes in the case of animals and fungi but in plants they are associated with the chloroplast. In Euglena gracilis the desaturases are located in the soluble fraction (Hithcock and Nichols, 1971; and Gurr, 1974).

The fatty acid substrate must be in the form of acyl-S-CoA, acyl-S-ACP or acyl-ester depending on the tissue type and desaturating enzyme. The form of the substrate in animals is acyl-CoA, in plants acyl-Sacyl carrier protein, and fungi use both acyl-S-CoA and acyl-O-ester lipid (Stumpf et al, 1975; Gurr and James, 1975; and Baker and Lynen, 1971).

Most desaturases are membrane bound and hence difficult to characterise in terms of individual components. However, in the systems most intensely studied, i.e. Euglena, rat liver, and hen liver, there is general agreement that the desaturase system has three components. These are cytochrome b_5, cytochrome b_5 reductase and a cyanide sensitive factor. The fully active desaturase can be reconstituted by combining all three components (Brenner, 1971; Gurr, 1974). The electron transport chain associated with fatty acid desaturation may be illustrated as follows (Gurr and James, 1975):



Desaturases have not been studied thoroughly and rules for classification have not been stated clearly. Nevertheless, it is generally believed that there are two types of desaturases, Δ and ω, and that there is a specific desaturase for a specific position on the fatty acid. The symbol Δ denotes that the enzyme is specific for a certain distance from the carboxyl end and ω indicates a specificity for a certain distance from the terminal methyl group. Δ-desaturases are characteristic of animals and ω-desaturases are present in plants (James, 1972; Hitchcock and Nichols, 1971; Gurr, 1974).

i. Factors Affecting Desaturation of Fatty Acids

The biosynthesis of unsaturated fatty acids by micro organisms is affected by aeration, temperature, culture age and carbon source concentration (Erwin, 1973). Either an increase in oxygen tension or a decrease in temperature will increase the unsaturated fatty acid content (Weete, 1974). This is explained in terms of the availability of O_2 which is a cofactor for desaturation reactions. As the culture ages, there is a tendency for certain unsaturated fatty acids to decrease (Deven and Manocha, 1976). In Choanephora cucurbitarum, there is a decrease in γ-linolenic acid (cis-6, cis-9, cis-12, octatrienoic acid) from 67% to 6% as the culture ages.

Studies with mammalian $\Delta 6$ desaturase showed that ATP enhances the desaturation of linoleic acid to γ-linolenic acid (Brenner, 1974). This effect is specific to ATP since no other nucleotide e.g. GTP, CTP, ADP or AMP had any effect on $\Delta 6$ desaturase activity. The rate of desaturation of linoleic acid to γ-linolenic acid, if plotted against linoleate concentration gives a sigmoidal curve. This implies that $\Delta 6$ desaturase is a regulatory enzyme whose positive effector is ATP. Thus, intracellular ATP levels may have a possible physiological significance in the production of γ-linolenic acid.

BIOLOGY OF CHOANEPHORA CUCURBITARUM

Choanephora cucurbitarum is a Phycomycetes belonging to the order mucorales (Alexopoulos, 1962). Most of the mucorales are saprobes living on dung and decaying plant or animal matter. A few mucorales are also parasites growing on other fungi and plants. Choanephora cucurbitarum causes rot of cucumbers and related fruits commonly isolated from decaying flowers of various kinds.

Phycomycetes are characterized by mycelium which forms a system of continuous tubes without crosswalls called septa. But septa do appear at the bases of the reproductive organs, sporangia or gametangia.

Choanephora cucurbitarum like other mucorales usually reproduce asexually by means of aplanospores. This fungus produces both multispored sporangia and monospore sporangiola called conidia. The conidial formation is favoured under lower temperature ($25^{\circ}C$), low humidity and low intensity light (Lilly and Barnett, 1953). Ultrastructure of the fungal cell shows mitochondrion, nucleus and other organelles characterized by the eukaryotic cells. This fungus is an obligate aerobe. Although very little is known about its metabolism, Phycomycetes are known to possess Embden-Myerof Pathway and Citric Acid Cycle (Ainsworth and Sussman 1965).

MATERIALS AND METHODS

Organism and Cultural Conditions

The fungus, Choanephora cucurbitarum (Berk. and Rav. Thaxter), was maintained in test tubes on sterilized solid medium consisting of 2.0% malt extract, 0.2% yeast extract and 2.0% Bacto agar adjusted to pH6.5. The slant cultures were incubated at 24°C ± 1°C in a Percival incubator (Boone, Iowa). The spore inoculum was obtained by growing the fungus on the same solid medium in 9 cm petri dishes under the optimum conditions for sporulation of low light intensity and low humidity. Spore suspension was prepared by dislodging the spores from 4-5 day old cultures with sterile distilled water. The suspension was centrifuged at 3000 rpm for 12 min. The supernatant was discarded and spores were resuspended in sterile distilled water. The above procedure was repeated to obtain a clean concentrated spore suspension of 10^5 spores/ml. The number of spores per ml was estimated using a hemocytometer.

All physiological studies were carried out in a liquid culture medium. The liquid culture media were of two types, synthetic and non-synthetic or complex. The complex medium contained 2.0% malt extract and 0.2% yeast extract in distilled water adjusted to pH6.5. The synthetic medium consisted of 20 gm of glucose, 2 gm of glutamic acid, 0.5 gm of $MgSO_4 \cdot 7H_2O$, 100 mg of thiamine, 1 ml of stock solution (Fe, Ca, Mn, Zn) made up to a litre in 0.1M K_2HPO_4/KH_2PO_4 buffer of pH6.5 (White and Powell, 1965). The nitrogen source in the synthetic medium was glutamic acid. In many experiments this was replaced with other amino acids or ammonium sulfate. The amount of these compounds used was calculated on the basis of nitrogen content present in 0.2% glutamic acid. When, in some experiments, more than one nitrogen source is used (e.g. when glutamic acid concentration is varied) the total

nitrogen content of the medium was kept constant. Details of media composition are described at appropriate places under the results section.

Fifty ml of liquid was contained in 250 ml Erlenmeyer flasks and there were at least five replicates for each treatment. The flasks containing media were autoclaved for 15 min at 15 lb/inch^2 of pressure. Each flask was inoculated with 0.5 ml of spore suspension. After a desired incubation period, the fungal mat was filtered through cheese cloth and washed with 500 ml of distilled water. Mycelium was weighed and a small portion was dried at 60^{o}C for 24 hrs for dry weight determination and the rest was used for further experimentation.

Lipid Extraction

Lipids were extracted by homogenizing the fungal mycelium in 20 volumes of chloroform:methanol (2:1, 1:1, 1:2) using a Virtis Homogenizer (Bligh and Dyers, 1959). The extract was filtered through defatted Whatman No. 1 filter paper into a separatory funnel. The extracted lipid was washed with 0.9% aqueous NaCl to remove any non-lipid contaminants (Folch et al, 1957). The lower organic phase was removed and dried using a Rotary Evaporator. The lipids were redissolved in petroleum ether and treated with anhydrous sodium sulfate to remove any residual water. The mixture of petroleum ether and lipids was used for preparation of fatty acid methyl esters. Reagents used for the investigation were redistilled analytical grade.

Esterification of Fatty Acids and Gas-Liquid Chromatography (GLC)

As fatty acids have high boiling points they have to be esterified prior to their analysis by gas-liquid chromatography. Total lipid extract was therefore dissolved in petroleum ether: acetone: 0.5N sodium methylate (1:1:5). The resulting mixture was acidified with 0.5 N hydrochloric acid and stored overnight at 4^{o}C (Manocha, 1975). The petroleum ether phase was

washed with ice cold distilled water and dried under nitrogen. The methyl esters were redissolved in n-hexane for injection into the Gas Chromatograph.

Fatty acid methyl esters were analysed using a Hewlett Packard 5700 A Gas-Liquid Chromatograph equipped Flame Ionization Detector and Fisher Recordall Series 5000 recorder fitted with an integrator. The 180 cm-3 mm glass column was packed with stationary phase, 10% silar 10c (Applied Science Laboratories Inc.) on solid support Chromosorb Q-AW-DMCS 100-120 mesh size. The flow rates of various gases were: H_2: 60 ml/min, N_2: 60 ml/min and air: 240 ml/min. The detector and injector port temperature were 250°C and 200°C respectively. The column temperature was programmed from 140°C - 200°C at 2°C/min or run isothermally at 150°C.

Fatty acids were identified by comparing the retention times with those of authentic standards (Applied Science Laboratories Inc.) and by GLC-Mass Spectrometry. The fatty acid (percent) were calculated using an electronic integrator.

Extraction of Amino Acids

The harvesting procedure was the same as described before except that the mycelium was washed with ice cold distilled water. A portion of the mycelium was used for dry weight determination and the rest for amino acid extraction.

Mycelium was immersed in roughly 10 times its volume of hot boiling water. The mixture was stirred briefly and boiled for 20 min (Bent and Morton, 1964). After extraction, the mixture was brought to room temperature, insoluble residues were filtered off and rinsed twice with the extractant. The extracts from different treatments were stored at 4°C for 6-8 hrs and centrifuged at 40,000 rpm for 2 hr using an International Preparative Ultacentrifuge. The supernatant was concentrated to about 2 ml using Rotary

Evaporator. The pH of the extract was adjusted to 2.0 with concentrated hydrochloric acid and then completely dried on a hot plate. The samples were sent out for analysis on Beckman 120 C single column Amino Acid Analyser. The amino acid mixture was redissolved in lithium citrate buffer for injection into the Amino Acid Analyser. The resin height of 9 mm (I.D.) column packed with UR30 cation resin was 58 cm. The column temperatures were 39°C for the first 220 min and 62.5°C for the remaining 235 min. During the first 220 min pH2.8 buffer, 0.3 N Li and 0.053 M citrate were used, whereas in the latter period of 235 min pH 4.1 buffer, 1.2 N Li and 0.21M citrate were employed. The flow rates of buffer and ninhydrin were 70 ml/hr and 35 ml/hr respectively.

Extraction of Proteins

Choanephora cucurbitarum was grown on glucose-ammonium sulfate medium for a desired time prior to a four hour incubation with [^{3}H] leucine (Amersham and Searle). After harvesting, the mycelium was subjected to amino acid extraction procedure as described above. The insoluble residue was homogenized in 20 volumes of 5% trichloracetic acid (TCA) at 4°C using a Virtis Homogenizer. The mixture was centrifuged at 5000 rpm for 15 min. The pellet was dissolved in 5 ml of 1N NaOH and centrifuged again (Letourneau et al, 1975). The supernatant was saved and the above procedure was repeated to obtain a total of 10 ml of NaOH extract. Aliquots of 0.1 ml of NaOH extract were dissolved in 15 ml of Aqueous Counting Scintillant (Amersham and Searle). Samples were analyzed using Searle Delta 300 liquid scintillation counter. Protein was determined according to the method of Lowry et al, (1951).

The metabolic inhibitors, cycloheximide (Sigma) prepared in 0.1 M potassium phosphate buffer pH6.5 and isophthalic acid (Eastman Chemical Co.) prepared in 95% ethanol were filtered sterilized.

RESULTS

I The Fatty Acid Composition of Choanephora cucurbitarum with and without Glutamic Acid

The fatty acid composition of the total cellular lipids of Choanephora cucurbitarum incubated at 24°C ± 1°C for 96 hrs on malt-yeast extract medium showed that palmitic (16:0) palmitoleic (16:1), stearic (18:0), and linoleic (18:2) acids were the major constituents. Linoleic acid was the predominant fatty acid. Small amounts of myristic (14:0) and oleic (18:1) and traces of pentadecanoic (15:0) and γ-linolenic (γ-18:3, cis-6, cis-9, cis-12 octatrienoate) acids were also detected (Table I: Fig. 3a). A similar fatty acid profile was observed using a synthetic medium of glucose-ammonium sulfate composition (Table I; Fig. 4a).

The fatty acid composition of Choanephora cucurbitarum incubated on either complex or synthetic media in the presence of glutamic acid showed that palmitic stearic, oleic, linoleic and γ-linolenic acids were the major fatty acids. Small amounts of myristic, pentadecanoic, palmitoleic, behenic (22:0), erucic (22:1), lignoceric (24:0) and cerotic (26:0) acids were also detected (Table I; Fig. 3b, 4B). The presence of glutamic acid in the growth medium consistently decreased the percent composition of linoleic acid and increased the percent composition of γ-linolenic acid. Glutamic acid also stimulated the biosynthesis of a new series of long chain fatty acids e.g. behenic (22:0) erucic (22:1), lignoceric (24:0) and cerotic (26:0) which comprise about 6% of the total fatty acid composition.

There was no difference in lipid yield under the experimental conditions used in these studies, with or without glutamic acid, but the addition of glutamic acid to either synthetic or complex media resulted in slight decrease in degree of unsaturation (Table I).

Table I Effect of Glutamic Acid on the Fatty Acid Composition of Choanephora cucurbitarum

Additions	Lipid Yield	Percent Fatty Acid Composition												Degree of Unsatn. Δ /Mole
		14:0	15:0	16:0	16:1	18:0	18:1	18:2	18:3	22:0	22:1	24:0	26:0	
Malt extract 2% + Yeast extract 2.0×10^{-1}%	210	1.3	T	17.4	8.4	12.4	1.1	61.0	T	ND	ND	ND	ND	1.29
Malt extract 2% Yeast extract 2.0×10^{-1}% Glutamate 2.0×10^{-1}% $(1.36 \times 10^{-2}$M)	223	1.1	T	23.4	1.7	20.2	19.1	13.0	15.4	0.4	2.7	1.3	1.7	0.96
Glucose 2% $(1.11 \times 10^{-1}$M) $(NH_4)_2SO_4$ 6.8×10^{-2}M	225	1.0	T	16.1	8.4	11.8	1.1	61.7	T	ND	ND	ND	ND	1.33
Glucose 2% $(1.11 \times 10^{-1}$M) $(NH_4)_2SO_4$ 6.8×10^{-2}M Glutamate 2.0×10^{-1}% $(1.11 \times 10^{-1}$M)	217	1.5	T	22.2	1.4	16.7	20.8	11.7	16.3	0.5	3.8	1.2	1.8	0.97

Lipid Yield is expressed as mg/g Dry Wt. of Mycelia

Δ /mole= [1.0 x (% Monoenes/100)] + [2.0 x (%Dienes/100)] + [3.0 x (% Trienes/100)]

T = Trace ND = Not Detected Harvest Time: 96 hrs.

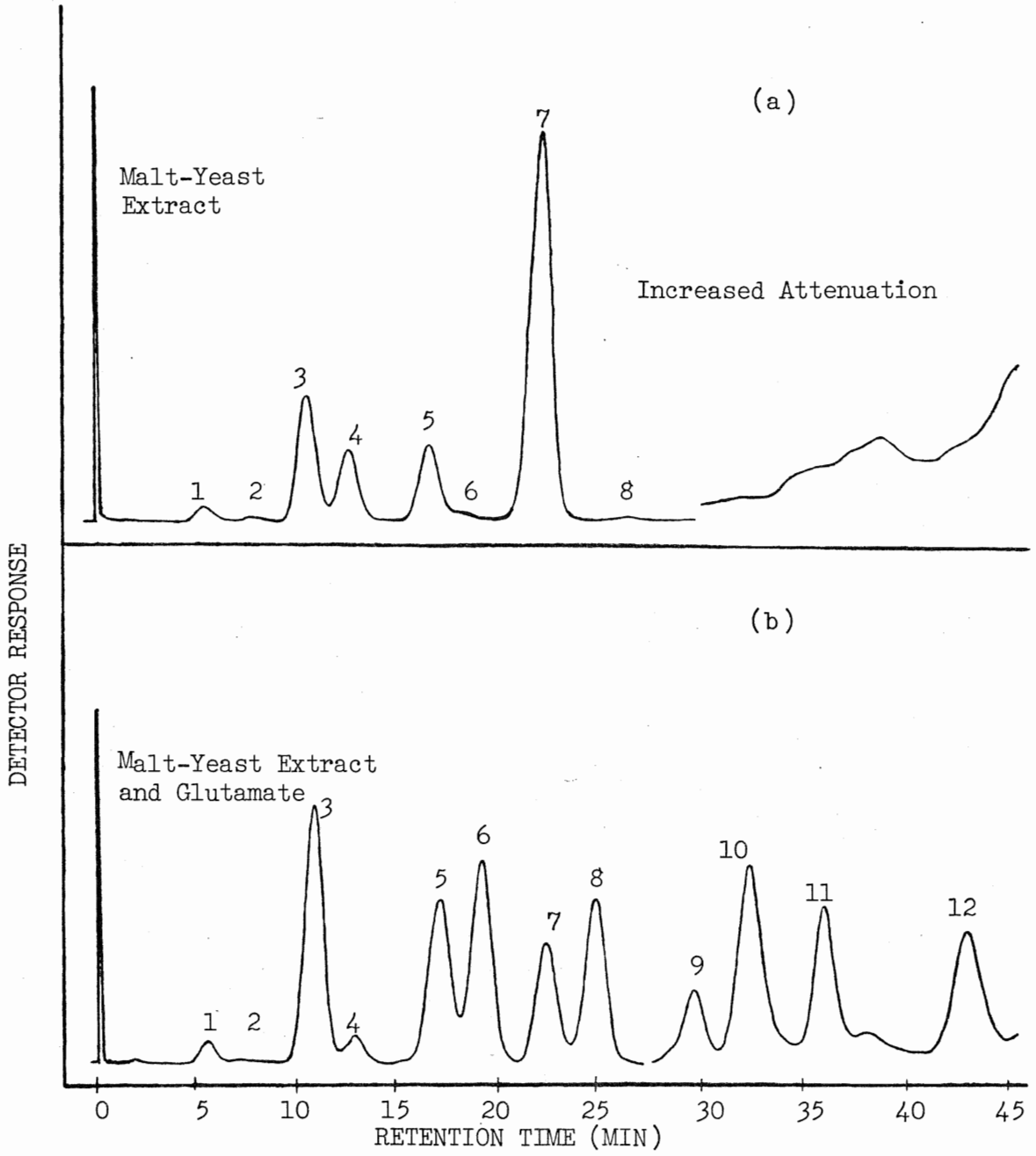


Fig. 3 Representative gas-liquid chromatogram of fatty acid methyl esters prepared from the lipid extracted from C. cucurbitarum grown on (a) malt yeast extract medium (b) malt yeast extract with glutamic acid medium for 96 hrs. Column dimensions: 180 cm x 3 mm; column packing: 10% Silar 10c on Chromosorb Q, AW-DMCS; column temperature 140-200°C (2°C/min); detector temperature 250° C; injector port temperature 200° C; N_2 flow, 60 ml/min; H_2 flow, 60 ml/min; air flow, 240 ml/min. Peaks 1-12 refer to: 1, $C_{14:0}$; 2, $C_{15:0}$; 3, $C_{16:0}$; 4, $C_{16:1}$; 5, $C_{18:0}$; 6, $C_{18:1}$; 7, $C_{18:2}$; 8, $C_{\gamma 18:3}$; 9, $C_{22:0}$; 10, $C_{22:1}$; 11, $C_{24:0}$; 12, $C_{26:0}$.

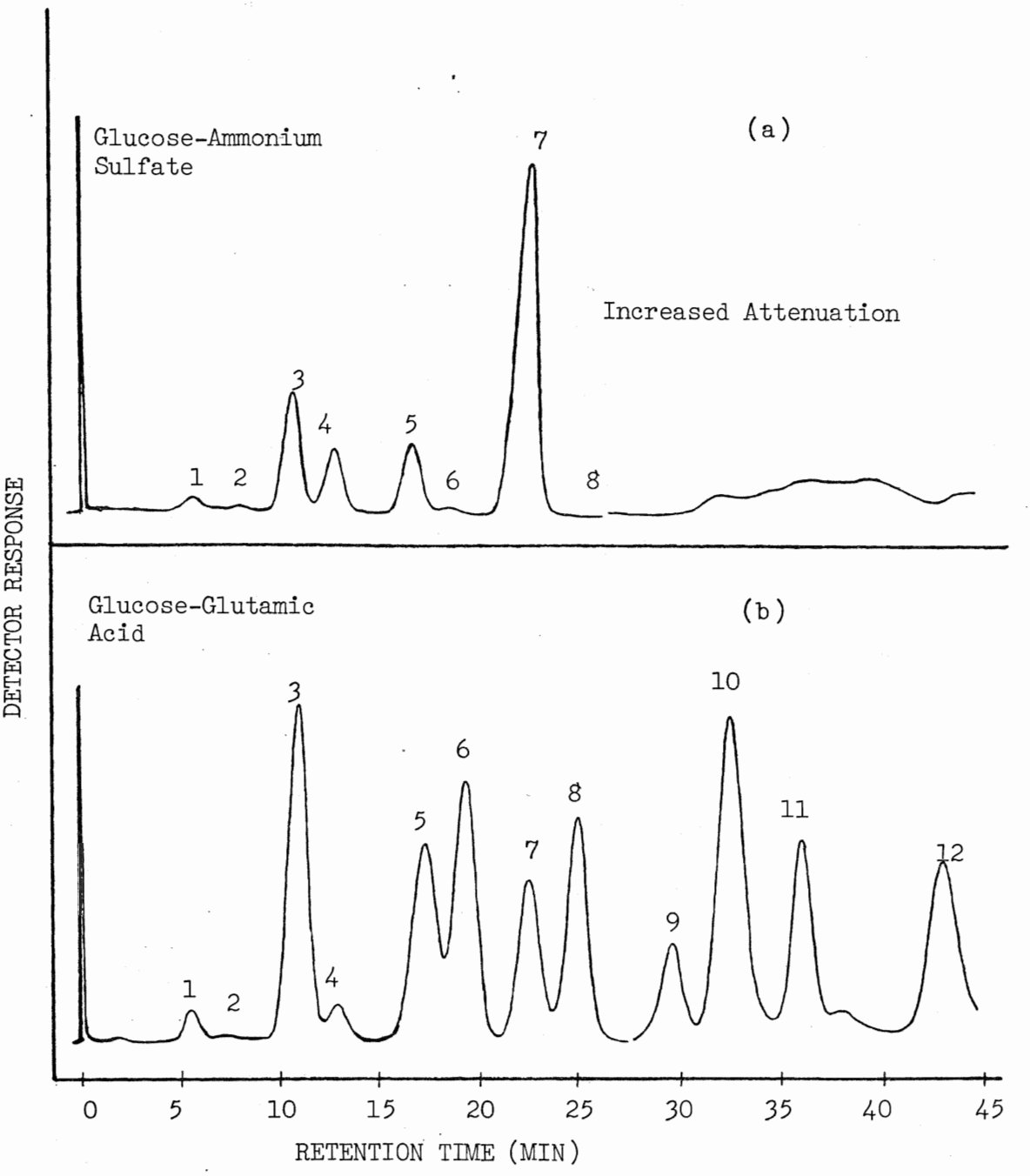


Fig. 4 Gas-liquid chromatogram of fatty acid methyl esters prepared from the lipid extracted from C. cucurbitarum grown on (a) glucose-ammonium sulfate medium (b) glucose-glutamic acid medium for 96 hrs. Column dimensions, 180 cm 3 3 mm; column packing: 10% silar 10c on Chromosorb Q, AW-DMCS; column temperature, 140-200° C (2° C/min); detector temperature 250° C; injection port temperature 200° C; N_2 flow, 60 ml/min; H_2 flow, 60 ml/min; air flow, 240 ml/min. Peaks 1-12 refer to: 1, $C_{14:0}$; 2, $C_{15:0}$; 3, $C_{16:0}$; 4, $C_{16:1}$; 5, $C_{18:0}$; 6, $C_{18:1}$; 7, $C_{18:2}$; 8, $C_{\gamma 18:3}$; 9, $C_{22:0}$; 10, $C_{22:1}$; 11, $C_{24:0}$; 12, $C_{26:0}$.

II Effect of Various Amino Acids on the Fatty Acid Composition of Choanephora Cucurbitarum

The possibility that other amino acids might duplicate the effect of glutamic acid on the fatty acid composition was considered. Therefore, the fungus was grown on synthetic medium of glucose-amino acids and incubated for 96 hrs at 24°C. The amino acids used were L-alanine, L-aspartic acid, L-arginine. L-methionine, L-phenylalanine, L-proline, L-leucine, L-serine and γ-aminobutyric acid. The amount of these compounds added to the medium was calculated on the basis of nitrogen equivalent present in 0.2% glutamic acid. Of the various amino acids used only proline and γ-aminobutyrate had the same effect on the lipid yield, degree of unsaturation and the fatty acid composition as that of glutamic acid. The effect of other amino acids (alanine, aspartate, mehtionine, leucine, arginine, phenylalanine and serine) on lipid yield, degree of unsaturation, and fatty acid composition resembled that of control which was free from any glutamic acid (Table II).

III Effect of Citric Acid Cycle Intermediates on the Fatty Acid Composition of Choanephora cucurbitarum

The problem of fatty acid elongation was approached from the point of view that the biosynthesis of long chain fatty acids is substrate limited. Glutamic acid provides the basic precursor of fatty acid biosynthesis, Acetyl-CoA and therefore results in the synthesis of long chain fatty acids. Hence various citric acid cycle intermediates were added to the synthetic medium of glucose-ammonium sulfate to observe their effect on the fatty acid composition. The amount of each compound used was calculated on the basis of carbon equivalent in 0.2% glutamic acid. All of the intermediates (malic acid, α- keto-glutaric acid, succinic acid and citric acid) and acetate had the same effect on the lipid yield, degree of unsaturation and fatty

Table II Effect of Various Amino Acids on the Fatty Acid Composition of Choanephora cucurbitarum

Amino Acid Used	Lipid Yield	Percent Fatty Acid Composition												Degree of Unsatn. △/Mole
		14:0	15:0	16:0	16:1	18:0	18:1	18:2	γ18:3	22:0	22:1	24:0	26:0	
Alanine 1.36×10^{-2}M	221	0.9	T	8.0	4.6	6.4	34.0	46.1	T	ND	ND	ND	ND	1.31
Aspartate 1.36×10^{-2}M	218	1.0	T	8.0	4.4	6.5	35.8	44.3	T	ND	ND	ND	ND	1.29
Methionine 1.36×10^{-2}M	253	0.9	T	8.1	4.5	5.9	35.1	44.0	T	ND	ND	ND	ND	1.28
Leucine 1.36×10^{-2}M	217	1.0	T	7.8	4.6	6.0	36.3	44.2	T	ND	ND	ND	ND	1.30
Arginine 3.40×10^{-3}M	245	0.8	T	7.6	4.5	5.3	36.1	45.8	T	ND	ND	ND	ND	1.32
Phenylalanine 1.36×10^{-2}M	236	0.6	T	7.3	4.4	4.4	35.1	48.0	T	ND	ND	ND	ND	1.36
Serine 1.36×10^{-2}M	234	1.0	T	7.3	4.4	5.1	35.6	46.7	T	ND	ND	ND	ND	1.33
Proline 1.36×10^{-2}M	212	1.7	T	23.8	3.6	7.8	31.2	12.4	13.5	0.3	1.4	1.5	2.9	1.02
Glutamate 1.36×10^{-2}M	229	1.5	T	23.3	3.3	8.7	31.9	12.1	13.0	0.4	1.2	1.5	3.1	1.00
γ-Amino Butyrate 1.70×10^{-2}M	245	1.5	T	28.7	2.9	8.2	30.3	10.5	12.3	0.5	1.5	1.3	2.6	0.93

Lipid Yield is expressed as mg/g Dry Wt. of Mycelia

$$\triangle/\text{mole} = [1.0 \times (\%\ \text{Monoenes}/100)] + [2.0 \times (\%\ \text{Dienes}/100)] + [3.0 \times (\%\ \text{Trienes}/100)]$$

T = Trace ND = Not Detected Harvest Time: 96 hrs.

Table III Effect of Citric Acid Cycle Intermediate on the Fatty Acid Composition of Choanephora cucurbitarum

Intermediate Used	Lipid Yield	Percent Fatty Acid Composition												Degree of Unsatn. △/mole
		14:0	15:0	16:0	16:1	18:0	18:1	18:2	γ18:3	22:0	22:1	24:0	26:0	
Acetate 3.4×10^{-2}M	231	1.5	T	28.0	2.9	8.1	30.0	11.4	12.2	0.4	1.6	1.2	2.7	0.94
Malate 1.70×10^{-2}M	256	1.6	T	27.5	3.1	7.9	30.2	11.1	12.4	0.5	1.7	1.4	2.6	0.94
α-Ketoglutarate 1.36×10^{-2}M	228	1.7	T	29.0	2.7	8.0	29.0	11.2	12.5	0.4	1.5	1.3	2.7	0.93
Succinate 1.70×10^{-2}M	247	1.4	T	28.5	2.8	8.3	29.5	11.5	12.1	0.4	1.4	1.4	2.7	0.93
Citrate 1.13×10^{-2}M	216	1.8	T	29.6	3.2	7.8	29.4	11.3	12.0	0.6	1.6	1.2	2.5	0.93
Glutamate 1.36×10^{-2}M	225	1.6	T	29.3	3.0	8.1	28.7	11.0	11.9	0.4	1.5	1.8	2.7	0.91

Lipid Yield is expressed as mg/g Dry Wt. of Mycelia

$$\triangle/\text{mole} = [1.0 \times (\%\ \text{Monoenes}/100)] + [2.0 \times (\%\ \text{Dienes}/100)] + [3.0 \times (\%\ \text{Trienes}/100)]$$

T = Trace

ND = Not Detected

Harvest Time: 96 hrs.

acid composition as that of glutamic acid (Table III).

IV Effect of Glutamic Acid Concentration on the Fatty Acid Composition of Choanephora cucurbitarum

Complex medium, which does not support the production of long chain fatty acids, has yeast extract as the nitrogen source. It is unreasonable to assume that yeast extract is free from any glutamic acid. Hence there must be minimum concentration of added glutamic acid which supports the production of detectable amounts of long chain fatty acids. In order to determine this Choanephora cucurbitarum was incubated on synthetic medium of glucose-ammonium sulfate-glutamic acid while varying the concentration of glutamic acid and ammonium sulfate to keep the total nitrogen contents of the medium at a constant value.

Employing a series of concentrations (5.0×10^{-1}% (3.4×10^{-2}M) -2×10^{-4}% (1.36×10^{-5} M)) of glutamic acid, the minimum concentration which stimulated the synthesis of detectable amounts of long chain fatty acids was found to be 3.0×10^{-4}% (2.04×10^{-5}M). The fatty acid profile in the presence of 2.0×10^{-4}% (1.36×10^{-5}M) resembled that of control, which was free from any glutamic acid. The concentrations (5.0×10^{-1}% (3.4×10^{-2}M) - 4.0×10^{-4}% (2.72×10^{-5}M)) exhibited a similar effect on the fatty acid composition, lipid yield, and the degree of unsaturation as in the case of 2.0×10^{-1}% (1.36×10^{-2}M) glutamic acid (Table IV).

V Amino Acid Pool Composition of Choanephora cucurbitarum with or without Long Chain Fatty Acids Stimulants

The amino acid pool of Choanephora cucurbitarum grown at $24^{\circ}C \pm 1^{\circ}C$ for 96 hrs on glucose-ammonium sulfate medium with or without long chain fatty acid stimulants showed that alanine, ornithine, glutamic acid and glycine were the

Table IV Effect of Glutamic Acid Concentration on the Fatty Acid Composition of Choanephora Cucurbitarum

Amount of Glutamate Used	Lipid Yield	Percent Fatty Acid Composition												Degree of Unsatn. △/mole
		14:0	15:0	16:0	16:1	18:0	18:1	18:2	18:3	22:0	22:1	24:9	26:0	
$5.0 \times 10^{-1}\%$ (3.4×10^{-2}M)	216	1.5	T	23.5	2.8	7.9	34.5	11.3	12.6	0.4	1.4	1.4	2.7	0.99
$2.0 \times 10^{-1}\%$ (1.36×10^{-2}M)	218	1.5	T	24.0	3.0	7.8	34.2	11.3	12.5	0.4	1.4	1.5	2.5	0.99
$2.0 \times 10^{-2}\%$ (1.36×10^{-3}M)	235	1.5	T	23.5	2.9	7.5	34.6	11.3	12.6	0.4	1.4	1.5	2.5	0.99
$2.0 \times 10^{-3}\%$ (1.36×10^{-4}M)	213	1.3	T	24.3	3.1	7.7	34.0	11.1	12.5	0.4	1.4	1.4	2.5	0.98
$1.0 \times 10^{-3}\%$ (6.80×10^{-5}M)	219	1.5	T	23.7	3.4	7.8	34.5	10.8	12.3	0.5	1.4	1.4	2.6	0.98
$5.0 \times 10^{-4}\%$ (3.40×10^{-5}M)	225	1.6	T	23.1	3.2	7.5	34.7	11.1	12.7	0.4	1.4	1.4	2.5	1.0
$4.0 \times 10^{-4}\%$ (2.72×10^{-5}M)	211	1.6	T	24.0	3.1	7.6	34.5	10.8	12.4	0.4	1.3	1.5	2.5	0.98
$3.0 \times 10^{-4}\%$ (2.04×10^{-5}M)	227	1.5	T	15.9	3.4	6.6	31.4	30.8	6.7	0.4	1.1	1.3	1.6	1.18
$2.0 \times 10^{-4}\%$ (1.36×10^{-5}M)	230	1.5	T	14.9	6.2	4.9	27.9	44.7	T	ND	ND	ND	ND	1.24
No Glutamate	217	0.8	T	7.9	4.2	3.8	32.1	50.6	T	ND	ND	ND	ND	1.38

Lipid Yield is expressed as mg/g Dry Wt. of Mycelia

△/mole = [1.0 x (% Monoenes/100] + [2.0 x (% Dienes/100)] + [3.0 x (% Trienese/100)]

T = Trace

ND = Not Detected

Harvest time: 96 hrs.

major free acids. The other amino acids present in small amounts were listed in Table V. This is in general agreement with Phipps and Barnett (1975).

The fact that small amounts of glutamic acid (27 μM) can support the production of long chain fatty acids led to the suggestion that glutamic acid but not its metabolic product may stimulate the synthesis of long chain fatty acids. Other compounds which also support the production of long chain fatty acids should increase the intracellular pool of glutamic acid. Hence the intracellular level of glutamic acid was analyzed with or without long chain fatty acid stimulants.

The amounts of free intracellular glutamic acid of Choanephora cucurbitarum incubated with and without glutamic acid were 18.7 and 3.4 μMoles per gm weight respectively. The free glutamic acid in the presence of other long chain fatty acid stimulants varied from 2.1 (succinate) to 9.3 (proline) μ Moles per gm dry weight (Table V). Thus all long chain fatty acid stimulants do not result in the increase of intracellular glutamic acid pool.

VI Effect of Glutamate Addition at Various Growth Times on the Fatty Acid Composition of Choanephora cucurbitarum

In all of the previous experiments C. cucurbitarum was grown on a medium containing glutamic acid during the entire incubation period of 96 hours. The possibility that shorter periods of glutamic acid incubation might stimulate the synthesis of long chain fatty acids was investigated.

When glutamate (27 μM) was added after 95 hrs of growth on synthetic medium of glucose-ammonium sulfate and the fungus harvested at 96 hrs, traces of long chain fatty acids were detected, but the percent linoleic and γ-linolenic acids were considerably less as compared with 96 hrs of incubation with glutamate.

Table V Amino Acid Pool Composition of Choanephora cucurbitarum with or without Long Chain Fatty Acids Stimulants

Additions	Asp	Thr	Ser	Asn	Gln	Pro	Glu	Gly	Ala	*AABA	Val	Met	Ile	Leu	Tyr	Phe	Orn	Lys	His	Arg
Acetate 3.4×10^{-2}M	0.8	1.4	0.7	1.8	3.4	3.2	4.6	3.7	33.6	0.3	1.4	0.1	0.8	0.9	0.9	0.5	5.8	0.8	1.2	0.9
Malate 1.7×10^{-2}M	0.7	0.8	0.4	2.5	0.7	6.3	3.5	1.3	31.5	0.5	1.8	0.3	0.5	0.5	0.6	0.4	4.0	1.5	2.1	0.8
α-Ketoglutarate 1.4×10^{-2}M	1.2	0.8	0.4	0.9	1.1	2.8	6.3	1.6	35.3	0.6	1.2	0.2	0.6	0.5	0.7	0.4	7.7	2.1	1.0	1.2
Succinate 1.7×10^{-2}M	1.5	1.5	0.5	2.4	0.4	5.3	2.1	1.7	29.8	0.7	2.2	0.5	0.9	1.0	1.0	0.6	5.6	2.6	0.7	1.5
Citrate 1.1×10^{-2}M	1.4	1.4	0.5	0.7	1.7	9.3	5.6	1.9	26.4	0.7	1.9	0.2	0.9	0.9	1.0	0.7	4.7	2.3	1.2	1.2
L-Proline 1.4×10^{-2}M	2.1	1.2	1.1	1.3	1.3	32.1	9.3	5.1	41.7	0.4	2.1	0.3	1.3	1.4	1.7	2.7	3.8	3.9	1.6	0.7
L-Glutamate 1.4×10^{-2}M	1.4	1.0	1.7	3.0	1.1	7.0	18.7	7.9	30.1	0.5	1.2	0.8	1.0	0.8	0.7	1.1	7.7	2.3	1.4	1.0
Control No Addition	0.8	1.8	0.4	2.1	0.3	2.2	3.4	1.2	25.4	0.5	1.1	0.2	0.6	0.6	0.6	0.3	5.3	1.7	0.8	0.6

Amino Acid Concentration is expressed as μMoles per gm dry weight

*AABA: α-Amino Butyric Acid

Harvest Time: 96 hrs.

The addition of glutamate after 72 hrs, 90 hrs, and 92 hrs of growth exhibited a similar fatty acid profile as is the case with a full 96 hrs of incubation in the presence of glutamic acid (Table VI).

VII Effect of Cycloheximide on the Fatty Acid Composition of Choanephora cucurbitarum

To test the possibility that glutamic acid might induce the synthesis of new protein(s) which is responsible for the biosynthesis of long chain fatty acids, glutamic acid was added in the growth medium along with the presence of cycloheximide. Two different concentrations and times of cycloheximide addition were used to demonstrate the adequate inhibition of $[^3H]$ leucine incorporation into proteins.

The fungus was grown for 91 hrs on glucose-ammonium sulfate medium prior to the addition of cycloheximide (2500 μg/ml and 100 μg/ml). Then, at 92 hrs 27.2 μM of glutamic acid and $[^3H]$ leucine were added to both treatments and the fungus was harvested at 96 hrs. The percent inhibition of $[^3H]$ leucine incorporation into proteins in the presence of 2500 μg/ml and 100 μg/ml of cycloheximide was 96.1 and 94.9 respectively. But, when cycloheximide (2500 μg/ml and 100 μg/ml), 27.2 μM glutamate and $[^3H]$ leucine were added simultaneously at 92 hrs, the percent inhibition was 91.7 and 81.9 respectively. This experiment clearly shows that cycloheximide concentration used inhibits cytoplasmic protein synthesis in Choanephora cucurbitarum by 92-96 per cent (Table VII).

The fatty acid composition of Choanephora cucurbitarum incubated for one hour with 100 μg/ml cycloheximide prior to its four hours of incubation with glutamate showed that the long chain fatty acids were produced as in the control situation whixh had only glutamic acid. The fatty acid composition in the presence of cycloheximide resembled that of the control

Table VI Effect of Glutamate Addition at Various Growth Times on Fatty Acid Composition of Choanephora cucurbitarum

Time of Glutamate (2.72×10^{-5}M) Addition	Lipid Yield	Percent Fatty Acid Composition												Degree of Unsatn. △/mole
		14:0	15:0	16:0	16:1	18:0	18:1	18:2	γ18:3	22:0	22:1	24:0	26:0	
0 hrs.	216	1.5	T	24.0	3.5	5.8	31.6	12.0	14.6	0.4	1.6	1.3	2.8	1.05
72 hrs.	208	1.5	T	24.3	3.6	7.1	30.3	12.1	14.6	0.4	1.8	1.5	2.9	1.04
90 hrs.	213	1.6	T	23.7	3.5	7.6	33.6	12.0	13.8	0.3	1.3	1.5	2.6	1.04
92 hrs.	240	1.4	T	24.7	3.0	7.7	31.2	11.4	13.3	0.4	1.3	1.5	2.5	0.98
94 hrs.	223	3.1	T	20.4	5.5	15.9	39.7	8.4	5.4	0.1	0.2	0.4	1.1	0.78
95 hrs.	219	4.2	T	13.8	6.6	19.2	48.8	6.2	1.3	T	T	T	T	0.71
95½ hrs.	237	4.3	T	11.3	6.2	16.4	56.1	5.7	T	ND	ND	ND	ND	0.74
No Glutamate	215	0.7	T	7.2	4.5	5.1	36.4	46.7	T	ND	ND	ND	ND	1.34

Lipid Yield is expressed as mg/g Dry Wt. of Mycelia

△/mole = [1.0 x (% Monoenes / 100)] + [2.0 x (% Dienes/100] + [3.0 x (% Trienes/100)]

T = Trace

ND = Not Detected

Harvest Time: 96 hrs.

Table VII Effect of Cycloheximide on $[^3H]$ Leucine Incorporation into Proteins

	$[^3H]$ Leu	$[^3H]$ Leu + 2500 μg/ml of cycloheximide added simultaneously	$[^3H]$ Leu + 100 μg/ml of cycloheximide added simultaneously	1 hr. preincubation with 2500 μg/ml cycloheximide plus $[^3H]$ Leu	1 hr. preincubation with 100 μg/ml cycloheximide plus $[^3H]$ Leu
dpm per mg dry weight	2.78×10^4	2.32×10^3	5.20×10^3	1.09×10^3	1.41×10^3
Percent inhibition of $[^3H]$ Leu incorporation into proteins by cycloheximide	——	91.7	81.9	96.1	94.9

Harvest Time: 96 hrs.

Table VIII Effect of Cycloheximide on the Fatty Acid Composition of Choanephora cucurbitarum

Additions after 92 hours	Lipid Yield	Percent Fatty Acid Composition												Degree of Unsatn. Δ/mole
		14:0	15:0	16:0	16:1	18:0	18:1	18:2	γ18:3	22:0	22:1	24:0	26:0	
No addition	195	1.3	T	16.2	9.5	11.2	0.8	60.9	T	ND	ND	ND	ND	1.32
Cycloheximide 100 μg/ml	216	0.9	T	19.5	6.6	11.3	1.3	60.4	T	ND	ND	ND	ND	1.29
Glutamate 27.2 μM	223	1.1	T	23.6	1.4	19.9	18.5	12.6	16.0	0.4	3.1	1.1	1.4	0.96
Glutamate 27.2 μM plus Cycloheximide 100 μg/ml	210	1.4	T	23.5	1.6	18.3	20.1	13.2	15.0	0.4	2.8	1.1	1.7	0.96

Lipid Yield is expressed as mg/g Dry wt. of Mycelia

$$\Delta/\text{mole} = \left[1.0 \times (\%\ \text{Monoenes}/100)\right] + \left[2.0 \times (\%\ \text{Dienes}/100)\right] + \left[3.0 \times (\%\ \text{Trienes}/100)\right]$$

T = Trace

ND = Not Detected

Harvest Time: 96 hrs.

which had no additions (inhibitor). Thus, it appears that five hours of cycloheximide incubation does not inhibit general fatty acid synthesis (Table VIII).

VIII Effect of Glucose Concentration on the Fatty Acid Composition of Choanephora cucurbitarum

Choanephora cucurbitarum was grown on glucose 1.0%, 2.0% or 3.0% in the presence of 0.2% glutamic acid. All three treatments showed the presence of the same amount ($\simeq$ 6%) of long chain fatty acids. The fungus was also grown on 2.0%, 3.0% or 4.0% glucose and ammonium sulfate which replaced glutamic acid in the above medium. The amount of ammonium sulfate was calculated on the basis of nitrogen equivalent in 0.2% glutamic acid. The growth medium of 2.0% glucose-ammonium sulfate showed the same profile as described earlier with no detectable long chain fatty acids. When growth medium of 3.0% or 4.0% glucose-ammonium sulfate was used, both treatments showed the presence of long chain fatty acids as with 0.2% glutamic acid, the amount of these long chain fatty acids were approximately 15% as compared with 6% in the presence of glutamic acid (Table IX).

The fatty acid profile of the lipid obtained from Choanephora cucurbitarum grown on a medium containing 1.0% glucose, 2.0% malt extract and 0.2% yeast extract showed traces of long chain fatty acids (Table IX).

IX Effect of Isophthalic Acid on the Fatty Acid Composition of Choanephora cucurbitarum in the presence of Glutamate and/or α-Ketoglutarate

The possibility that glutamic acid is oxidised to α-ketoglutarate prior to stimulating the synthesis of long chain fatty acids was considered. Therefore, fungus was incubated with isophthalate which is an inhibitor of glutamate dehydrogenase.

Choanephora cucurbitarum was grown for 91½ hrs on glucose-ammonium sulphate medium prior to the addition of isophthalic acid (2.72 µM, 27.2 µM,

Table IX Effect of Glucose Concentration on the Fatty Acid Composition of Choanephora cucurbitarum

Additions	Lipid Yield	Percent Fatty Acid Composition												Degree of Unsatn. Δ/mole
		14:0	15:0	16:0	16:1	18:0	18:1	18:2	γ18:3	22:0	22:1	24:0	26:0	
Glucose 2% (1.11×10^{-1}M) + Glutamate 2.0×10^{-1}% (1.36×10^{-2}M)	215	1.4	T	23.4	1.5	20.2	18.6	12.8	15.7	0.4	2.8	1.4	1.6	0.95
Glucose 3% (1.67×10^{-1}M) + Glutamate 2.0×10^{-1}% (1.36×10^{-2}M)	243	1.2	T	23.9	1.4	19.9	18.3	12.9	16.2	0.5	2.8	1.2	1.7	0.97
Glucose 1% (5.56×10^{-2}M) + Glutamate 2.0×10^{-1}% (1.36×10^{-2}M)	219	1.3	T	23.6	1.5	20.0	18.5	12.8	16.0	0.4	2.8	1.3	1.7	0.96
Glucose 2% (1.11×10^{-1}M) + $(NH_4)_2SO_4$ 6.8×10^{-2}M	206	1.1	T	17.5	7.5	11.8	1.0	61.0	T	ND	ND	ND	ND	1.29
Glucose 3% (1.67×10^{-1}M) + $(NH_4)_2SO_4$ 6.8×10^{-2}M	216	1.5	T	19.2	1.4	15.9	17.9	13.2	15.5	1.2	7.3	3.5	3.8	1.00

This Table is Continued on Next Page

Table IX Effect of Glucose Concentration on the Fatty Acid Composition of Choanephora cucurbitarum Contd.

Additions	Lipid Yields	Percent Fatty Acid Compositions											Degree of Unsatn. △/mole
		14:0	15:0	16:0	16:1	18:0	18:1	18:2	γ18:3	22:0	22:1	24:0	26:0	
Glucose 4% (2.22×10^{-1}M) + $(NH_4)_2SO_4$ 6.8×10^{-2}M	228	1.3	T	20.5	1.9	17.3	16.4	11.4	12.3	1,3	9.5	4.3	3.6	0.88
Glucose 1% (5.56×10^{-2}M) + Malt extract 2% + Yeast extract 2.0×10^{-1}%	248	1.2	T	15.8	6.7	13.3	3.2	58.3	1.5	T	T	T	T	1.30

Lipid Yield is expressed as mg/g Dry Wt. of Mycelia

△/mole = [1.0 x (% Monoenes/100] + [2.0 x (% Dienes/100)] + [3.0 x (% Trienes/100)]

T = Trace

ND = Not Detected

Harvest Time: 96 hrs.

Table X Effect of Isophthalate Concentration on the Fatty Acid Composition of Choanephora cucurbitarum

Additions after 92 hours	Lipid Yield	Percent Fatty Acid Composition												Degree of Unsatn. △/mole
		14:0	15:0	16:0	16:1	18:0	18:1	18:2	γ18:3	22:0	22:1	24:0	26:0	
No Additions	217	0.8	T	14.5	8.7	12.3	1.1	62.6	T	ND	ND	ND	ND	1.35
Glutamate 27.2 µM	208	1.3	T	24.0	1.5	19.2	19.3	12.9	15.6	0.3	2.7	1.4	1.6	0.96
Isophthalate 2.72 µM + Glu 27.2 µM	223	1.5	T	22.8	1.6	21.2	18.0	12.6	15.9	0.5	2.9	1.3	1.7	0.95
Isophthalate 27.2 µM + Glu 27.2 µM	205	1.8	T	22.5	1.4	18.4	20.6	13.5	15.3	0.4	3.2	1.3	1.6	0.98
Isophthalate 272 µM + Glu 27.2 µM	186	1.1	T	16.4	7.7	12.2	1.1	61.5	T	ND	ND	ND	ND	1.30
Isophthalate 272 µM	194	1.1	T	18.7	7.3	11.5	0.9	60.5	T	ND	ND)	ND	ND	1.29

Lipid Yield is expressed as mg/g Dry Wt. of Mycelia

$$\triangle/\text{mole} = [1.0 \times (\%\ \text{Monoenes}/100)] + [2.0 \times (\%\ \text{Dienes}/100)] + [3.0 \times (\%\ \text{Trienes})]$$

T = Trace

ND = Not Detected

Harvest Time: 96 hrs.

Table XI Effect of Isophthalate on Fatty Acid Composition in Presence of Glutamate and/or ∝-Ketoglutarate

Additions after 92 Hours	Lipid Yield	Percent Fatty Acid Composition												Degree of Unsatn. △/Mole
		14:0	15:0	16:0	16:1	18:0	18:1	18:2	γ18:3	22:0	22:1	24:0	26:0	
No Addition	210	1.0	T	16.8	8.5	11.6	1.0	61.1	T	ND	ND	ND	ND	1.32
Glutamate 27.2 µM	197	1.0	T	21.8	1.9	20.0	20.6	12.0	16.2	0.6	2.8	1.2	2.0	0.98
Glutamate 27.2 µM + Isophthalate 272 µM	203	0.8	T	17.2	8.1	11.3	1.0	61.1	T	ND	ND	ND	ND	1.32
∝-Ketoglutarate 27.2 µM	243	1.3	T	25.0	2.3	15.0	20.9	12.6	16.4	0.5	2.9	1.2	2.0	1.01
∝-Ketoglutarate 27.2 µM + Isophthalate 272 µM	215	1.1	T	21.9	1.4	16.1	19.1	10.4	17.4	0.6	2.8	1.1	1.9	0.96
27.2 µM ∝-KG + 27.2 µM Glu + Isophthalate 272 µM	186	1.1	T	21.4	1.5	19.8	19.1	11.6	19.4	0.6	2.6	1.0	1.9	1.05
Isophthalate 272 µM	204	0.9	T	15.3	8.4	12.0	1.2	62.2	T	ND	ND	ND	ND	1.34

Lipid Yield is expressed as mg/g Dry Wt. of Mycelia

△/mole = [1.0 x (% Monoenes/100)] + [2.0 x (% Dienes/100)] + [3.0 x (% Trienese)]

T = Trace

ND = Not Detected

Harvest Time: 96 hrs.

272 µM). Then, at 92 hrs 27.2 µM of glutamic acid was added to each of the three incubations and the fungus was harvested at 96 hrs. The fatty acid profile with 2.72 µM isophthalate plus 27.2 µM glutamic acid and 27.2 µ M isophthalate plus 27.2 µM glutamic acid was the same as 27.2 µM glutamate alone. But with 272 µM isophthalate plus glutamate the fatty acid profile was the same as that of the control which was free from any glutamic acid. It is noteworthy that 272 µM isophthalate alone did not affect the fatty acid composition. It was the same as in the control which had no additions (Table X; Fig. 5).

A further consideration of the above mentioned possibility; α-ketoglutarate was added together with isophthalic acid. The fatty profile of Choanephora cucurbitarum incubated for one-half hour with 272 µ M isophthalic acid prior to its 4 hr incubation with 27.2 µM glutamate plus 27.2 µM α-ketoglutarate showed the presence of long chain fatty acids. In addition, the fatty acid profile with 272 µM isophthalic acid plus 27.2 µM α-ketoglutarate was the same as with 27.2 µM glutamate alone (Table XI).

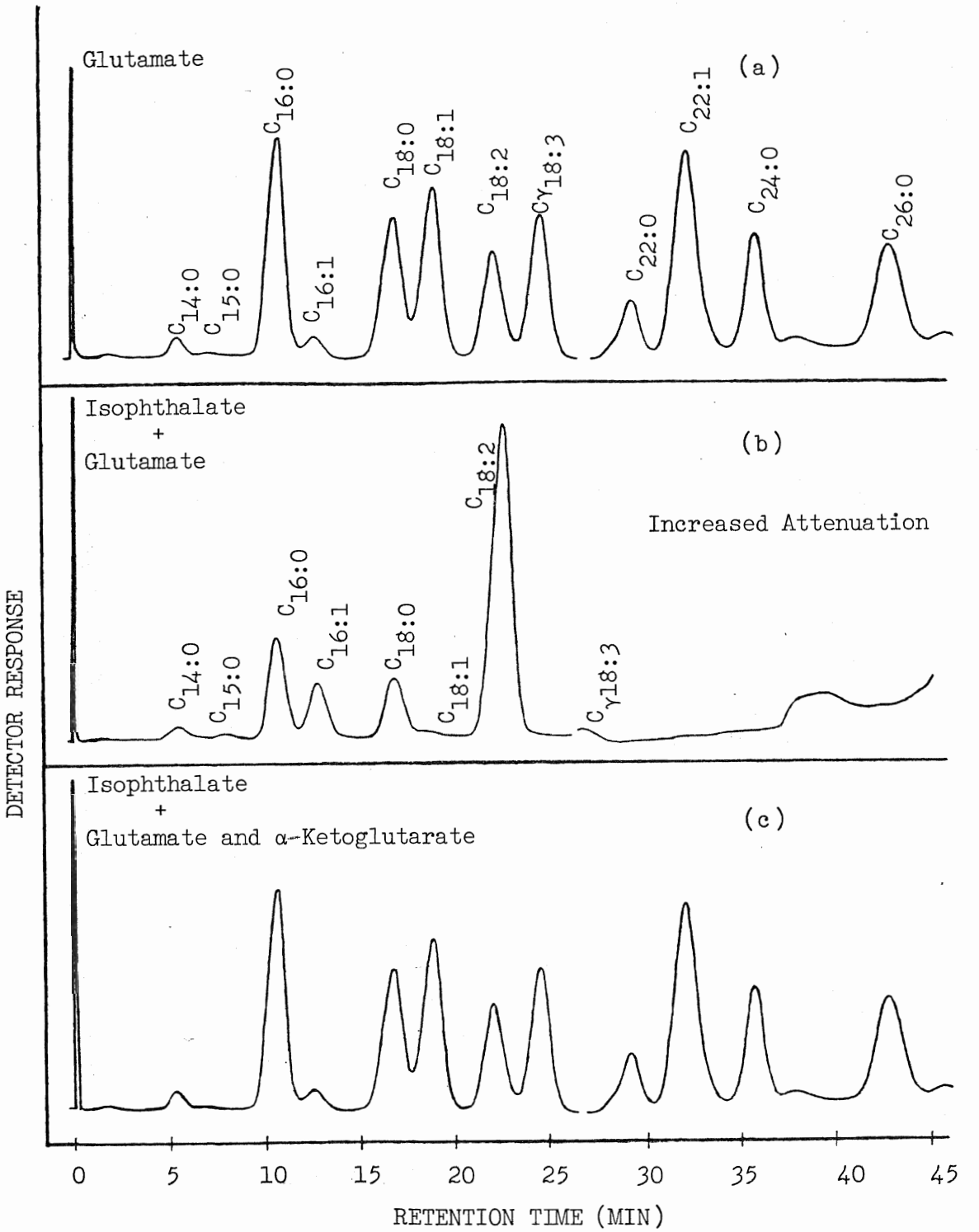


Fig. 5 Gas-liquid chromatogram of fatty acid methyl esters prepared from lipid extracted from 96 hr old culture of C. cucurbitarum grown on glucose ammonium sulfate medium with further additions of (a) glutamate (27.2 µM) at 92 hr of growth (b) isophthalic acid (272 µM) at 91½ hr and glutamate (27.2 µM) at 92 hr of growth and (c) isophthalate (272 µM) at 91½ hr plus glutamate (27.2 µM) and α Ketoglutarate (27.2 µM) at 92 hr of growth. The conditions used for the operation of gas-liquid chromatograph were the same as described in Figs. 3 and 4.

DISCUSSION

The presence of glutamic acid in the growth medium decreased the percent linolenic acid, increased the percent γ-linolenic acid and stimulated the production of long chain fatty acids (Table 1). This result is in agreement with the finding of Deven and Manocha (1975) that glutamic acid induces the appearance of long chain fatty acids. But, in contrast to the present study, the above authors did not detect any changes in the percent linoleic acid and the γ-linolenic acids associated with the addition of glutamic acid to the growth medium. This discrepancy may be due to differences in culture conditions employed in the two investigations.

The fatty acid composition of most micro-organisms is influenced by the culture age (Erwin, 1973). Deven and Manocha, (1976) reported percent γ-linolenic acid decreased from 67% to 6% as the culture aged from 24 hrs to 72 hrs respectively. Under the experimental conditions used in this study of 96 hrs harvest time, γ-linolenic acid decreased to a trace amount. The fatty acid profiles of Choanephora cucurbitarum grown without glutamic acid for 30 hrs and 96 hrs were significantly different. The 96 hr old cultures showed that palmitic, palmitoleic, stearic and linoleic acids were the major constituents. Whereas, 30 hr old cultures showed palmitic, oleic, linoleic, and γ-linolenic acids as the major constituents (Deven and Manocha, 1975). The differences in effect of glutamic acid on the fatty acid composition reported in the two studies may thus be explained on the basis of cultural age.

Glutamic acid stimulates the synthesis of long chain fatty acids regardless of age of mycelia used. Various ways in which glutamic acid may stimulate the synthesis of long chain fatty acids are given on page VIII. These may

be simplified to three hypotheses:

1) glutamic acid produces the substrate for fatty acid synthesis, Acetyl-CoA
2) glutamic acid acts as an inducer to produce a new protein which is responsible for synthesis of long chain fatty acids.
3) glutamic acid has some specific stimulatory role such as the production of a rate limiting factor for fatty acid synthesis.

These possible roles of glutamic acid in the biosynthesis of long chain fatty acids are discussed in this section.

1. Substrate-C_2 Unit Hypothesis

This hypothesis states that biosynthesis of long chain fatty acids is substrate (acetyl-CoA) limited. The addition of glutamic acid increases the level of acetyl-CoA pool and consequently, the synthesis of long chain fatty acids.

Glutamic acid may be deaminated to α-ketoglutarate or decarboxylated to γ-aminobutyric acid which can then be converted to succinic-semialdehyde. Both of these compounds (α-ketoglutarate and succinyl-semialdehyde) can be oxidized to yield acetyl-CoA (White et al, 1973). It has been demonstrated that mammalian tissue can incorporate glutamate carbon into fatty acids (Madsen and Chaikoff, 1964). But it has not been shown whether glutamic acid carbon goes preferentially to long chain or short chain fatty acids.

The pattern of fatty acids produced is influenced by the concentration of malonyl-CoA both in plants (Bartley et al, 1967) and in animals (Smith and Dils, 1966). Studies in vitro indicate that when high concentrations of malonyl-CoA are used, there appears a new fatty acid (C_{18}) which was never observed under any experimental conditions employed (Smith and Dils, 1966). There is some evidence that glutamic acid may produce acetyl-CoA which may be carboxylated to yield malonyl-CoA, and stimulate the synthesis of long

chain fatty acids. One test for this hypothesis would be to incubate the fungus with compounds which increase the level of acetyl-CoA. Compounds used in this stydy includes the citric acid cycle intermediates, α-ketoglutarate, succinate, citrate, malate, acetate itself, increased glucose concentration and the amino acids γ-aminobutyric acid, proline, aspartic acid, alanine, methionine, leucine, arginine, phenylalanine and serine. The fact that acetate, the tricarboxylic acid cycle intermediates proline, γ-aminobutyric acid and increased glucose concentration can all stimulate the production of long chain fatty acids tends to support the contention that the biosynthesis of long chain acids is substrate (acetyl-CoA) limited. But aspartic acid and alanine did not support the production of long chain fatty acids, although both can be oxidized to yield acetyl-CoA. This observation does not support the above hypothesis.

A more serious objection to this hypothesis is the minimum concentration of glutamic acid required to stimulate the synthesis of long chain fatty acids. When 3.0×10^{-4} percent (13 μM) glutamate was used detectable amounts of long chain fatty acids were present, and in the presence of 4.0×10^{-4} percent (27 μM) glutamate, the long chain fatty acids comprised 6 percent of the total, as much as when 0.5 percent (3.4×10^{-2}M) glutamic acid was used. Although the concentration of glutamic acid was decreased 1000 fold the percentage of long chain fatty acids and the lipid yield remained constant (Table IV). If glutamate were providing just the substrate-C_2 unit for long chain fatty acid synthesis then one would expect a decrease in either the lipid yield or percent long chain fatty acids when the amount of glutamate (27 μM) can support the production of long chain fatty acids is difficult to explain on the basis of this hypothesis.

2. Inducer Hypothesis

Some other peculiarities of glutamic acid metabolism are known in fungi. Bacon et al, (1975) reported that glutamic acid stimulated the biosynthesis of a mycotoxin in Aspergillus ochraceus. Proline was the only amino acid which could duplicate the effect of glutamic acid. Furthermore, this effect was most pronounced when the fungus was incubated with glutamic acid at early stages of development. In fact, this stimulatory effect of glutamic acid disappeared if it is added after 72 hours of fungal growth. Bacon et al (1975) postulated that the mechanism of stimulatory effect of glutamic acid was related to fungal development and RNA synthesis.

Such a hypothesis cannot explain the effect of glutamic acid on fatty acid composition in Choanephora cucurbitarum since long chain fatty acids are detected even though the fungus is incubated with glutamate for only one hour of growth (Table VI).

However, it is of interest to know whether glutamic acid or its metabolic product induces the production of a new protein which is responsible for synthesis of long chain fatty acids. It was demonstrated that glutamic acid supports the synthesis of long chain fatty acids even though more than 90 percent of cytoplasmic protein synthesis is arrested by the addition of cycloheximide (Tables VII, VIII). Thus it appears that the biosynthetic machinery for production of long chain fatty acids is present in the fungal cell prior to addition of glutamic acid.

In summary, the enhancement of the fatty acid elongation resulting from the addition of glutamic acid is unlikely to be due to induction of a new protein or simply increased level of acetyl-CoA.

3. Specific Stimulatory Role Hypothesis

The presence of a small amount of glutamic acid (27 μM) can result in

the synthesis of long chain fatty acids. This suggests that glutamic acid has a specific role rather than a general effect as stated by the substrate-C_2 unit hypothesis. A corollary is that all of the other compounds which also support the production of long chain fatty acids should increase the intracellular level of glutamic acid.

The amount of free intracellular glutamic acid in the absence of any long chain fatty acid stimulants and in the presence of glutamate was 3.4 and 18.7 μMoles per gram dry weight. The free intracellular glutamic acid in the presence of succinate which also stimulates the production of long chain fatty acids was 2.1 μMoles per gram dry weight (Table V). The apparent lack of correlation between free intracellular glumac acid with and without long chain fatty acids stimulants tends to negate the above suggestion.

Hence, even though a small amount of glutamic acid does support the production of long chain fatty acids, the effect may not be directly due to glutamic acid. Thus one can infer that it is not glutamic acid but a metabolic product which is responsible for stimulating the synthesis of long chain fatty acids.

As stated earlier, citric acid cycle intermediates and increased glucose concentration can support the production of detectable amounts of long chain fatty acids. This indicates that glutamic acid may have to be oxidized to α-ketoglutarate before stimulation of long chain fatty acids production occurs in Choanephora cucurbitarum.

$$\text{Glutamate} + \text{NAD(P)}^+ + H_2O \xrightarrow[\text{Dehydrogenase}]{\text{Glutamate}} \alpha\text{-ketoglutarate} + NH_4^+ + H^+ + \text{NAD(P)H}$$

The Phycomycetes glutamate dehydrogenase (GDH) is NAD linked and present in the cytoplasm. The Michaelis-Menten binding constants (Km) for glutamate with GDH of Achlya, Pythium, and Neurospora crassa are 3.1×10^{-3}M, 1.7 x

10^{-3}M and 5.6 x 10^{-3}M respectively. Isophthalic acid is a competitive inhibitor of oxidative deamination of glutamic acid. The Ki for bovine GDH is 5.6 x 10^{-4}M whereas in the case of Neurospora crassa it is 6.1 x 10^{-5}M, (Smith, et al, 1975).

The addition of 27 μM glutamic acid can stimulate the synthesis of long chain fatty acids in Choanephora cucurbitarum. This stimulation is abolished if isophthalic acid (272 μM) is also added to the incubation mixture. The oxidation of glutamic acid is probably inhibited. But α-ketoglutarate (27 μM) can stimulate the synthesis of long chain fatty acids in the presence of glutamate (27 μM) and isophthalic acid (272 μM) supporting the present suggestion (Tables X, Xi).

Some Speculations

It is important to realize that one cannot expect to obtain unequivocal answers at the molecular level when working with whole cells. This study provides indirect evidence for events occuring at the molecular level. Hence it would be erroneous to make conclusive statements as to the mechanism whereby glutamic acid stimulates the synthesis of long chain fatty acids.

Studies with yeast fatty acid synthetase indicate that the fatty acid possessing the maximum number of carbon atoms is stearate (C_{18}) (Weete, 1974), (Sumper et al, 1969). The fatty acids having chain length > C_{18} carbon atoms are the result of the elongating enzymes. These enzymes are associated with the mitochondrion and endoplasmic reticulum (Hitchcock and Nichols, 1971).

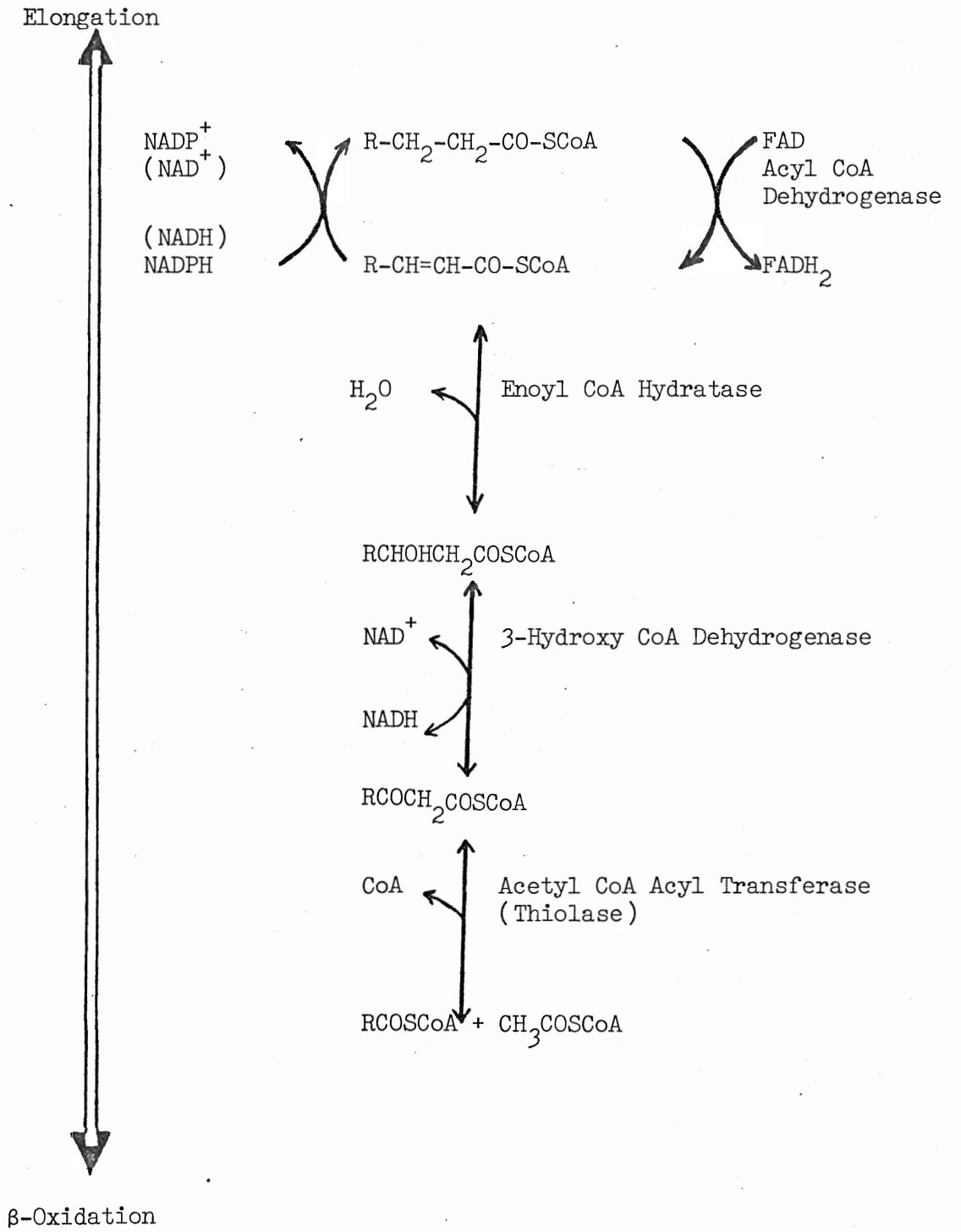
There are two distinct sites for elongating pre-existing fatty acids: mitochondrial and microsomal. The work on mammalian microsomal elongation system indicates that this pathway functions to synthesize proper substrate for desaturating enzymes by elongating the existing unsaturated fatty acids.

In fact, synthesis of arachidonic acid from γ-linolenic acid necessitates the addition of the C_2 unit prior to the final desaturation. The substrates which show optimal activity with microsomal elongating enzymes are unsaturated. Thus its products are also unsaturated fatty acids (Nugteren, 1965; Seubert and Podack, 1973).

Mitochondrial elongation synthesizes mainly saturated fatty acids although this pathway can also elongate unsaturated fatty acids (Seubert and Podack, 1973). The fact that the long chain fatty acids present in Choanephora cucurbitarum are mostly saturated fatty acids suggests that these acids are products of the acetyl-CoA dependent elongation system. Therefore, it is not unreasonable to assume that compounds which stimulate the production of long chain fatty acids in Choanephora cucurbitarum should provide some factor(s) for the mitochondrial elongation process.

The mechanism of acetyl-CoA dependent elongation is basically the reversal of β-oxidation (Fig. 6) (Whereat, 1971; Seubert and Podack, 1973). There are two types of mitochondrial elongation; heart type (NAD linked) and liver type (NADPH-linked). Hinsch et al, 1976; Shimakata et al, 1977). The rate limiting factors are NADH or NADPH depending upon the tissue type. The compounds which increase the intra-mitochondrial NADH or NADPH enhance the mitochondrial elongation reaction. The substrates affecting NADH level are succinate and other citric acid cycle intermediates. Whereas those affecting the level of NADPH are citrate, isocitrate, glutamate, α-ketoglutarate, malate, fumarate but not succinate (Hinsch et al, 1976). In Choanephora cucurbitarum the compounds which stimulate the synthesis of long chain fatty acids are succinate, fumaric acid, malate, citrate, and α-ketoglutarate, γ-aminobutyric acid, glutamic acid, proline and increased glucose concentration. It is conceivable that all of these compounds can be oxidized to yield the rate limiting factor for mitochondrial elongation, NADH.

Fig. 6 Relationship between β-oxidation and elongation of fatty acids
Shimakata et al (1977)



Glucose can be converted to pyruvate through the Embden-Myerhof pathway. Pyruvate can be decarboxylated to yield acetyl-CoA which can enter Krebs cycle and be oxidized to give NADH (Lehninger, 1975). This study provides some evidence that glutamic acid may be oxidized to α-ketoglutarate prior to stimulation of long chain fatty acids. Hence, glutamate could provide the rate limiting factor NADH for synthesis of long chain fatty acids. Proline could be converted to glutamic acid via pyrroline-5-carboxylic acid pathway as reported in many micro-organisms (Bacon, et al, 1975). The intramitochondrial NADH level can be also increased by γ-aminobutyric acid since it can be converted to succinate (White et al, 1973)

The basic assumption for this hypothesis is that all the long chain fatty acids producing stimulants increase the NADH:NAD^+ ratio. Aspartic acid and alanine can also be shuttled through the citric acid cycle, yet they do not stimulate the synthesis of long chain fatty acids. This presents a serious objection to the hypothesis that long chain fatty acids stimulants increase intramitochondrial NADH. However, this hypothesis does provide an explanation for most of the data.

NADH-dependent mitochondrial elongation proceeds in the presence of a rate limiting factor, NADH. The compounds which support the synthesis of long chain fatty acids increase the intramitochondrial NADH (Whereat, 1971) Seubert and Podack, 1973). The primary metabolic fate of NADH is to synthesize ATP via oxidative phosphorylation. But under the conditions of high phosphate potential, NADH is used to synthesize long chain fatty acids (Hinsch et al, 1976). Thus, the function of NADH dependent mitochondrial elongation is to synthesize long chain fatty acids for the purpose of conserving reducing equivalents. If a similar mechanism was operating in Choanephora cucurbitarum then one would expect that long chain fatty acids are preferentially located

in the fuel storage lipids. In fact, the majority of the long chain fatty acids are associated with the neutral lipids (Deven and Manocha, 1975).

This hypothesis also provides an explanation for the fact that there is a decrease in linoleic acid and increase in γ-linolenic acid in the presence of long chain fatty acid stimulants. As stated in the above paragraph, NADH dependent mitochondrial elongation proceeds under the conditions of high phosphate potential i.e. high intracellular ATP. Studies with mammalian Δ 6 desaturates showed that ATP enhances the desaturation of linoleic acid to γ-linolenic acid and this enhancement followed a sigmoidal curve. Furthermore, this effect is specific to ATP since no other nucleotide e.g. GTP, CTP, ADP or AMP had any effect on $\Delta 6$ desaturase activity (Brenner, 1974). Brenner's studies may explain the finding that long chain fatty acid stimulants increase γ-linolenic acid and decreases linoleic acid.

These observations suggest that intramitochondrial NADH could influence the fatty acid composition of Choanephora cucurbitarum. To pursue this, it would be useful to ascertain whether long chain fatty acids present in Choanephora cucurbitarum are synthesized from existing fatty acids or are the result of de novo synthesis. It would also be helpful to know whether microsomal or mitochondrial enzymes are involved in the synthesis of long chain fatty acids. Finally, a correlation is needed between reduced nucleotide and appearance of long chain fatty acids in the presence of long chain fatty acid stimulants.

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APPENDIX I

All experiments, the results of which are shown in tables, were repeated twice except V, VII and XI.

Experiment V (Table V) was performed only once, but the mycelia from which amino acids were extracted were further analysed for its fatty acid composition. This showed that the control had no long chain fatty acids in contrast to acetate, malate, α-ketoglutarate, succinate, citrate, L-proline and L-glutamic acid supported the production of long chain fatty acids.

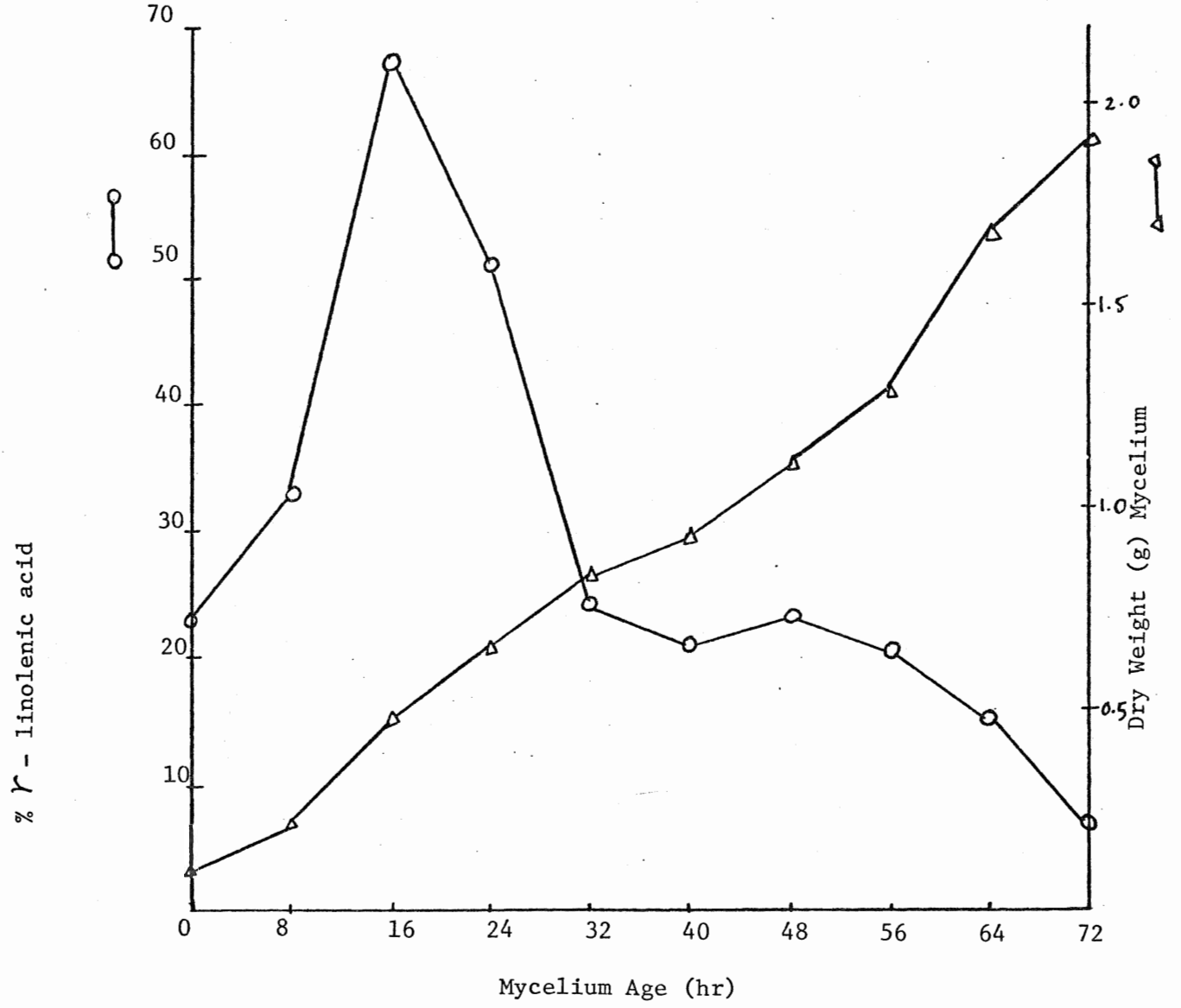
Experiment VII (Table VII) was performed once but the control i.e. incorporation of [^{3}H] Leu into proteins without cycloheximide was duplicated.

Experiment XI (Table XI) was repeated three times.

Mass spectroscopic analysis of proline showed that it had no glutamic acid contamination.

APPENDIX II

The effect of age of culture on γ - linolenic acid production and total dry weight of the mycelium (Deven, 1976)



APPENDIX III

The detector response was not calibrated. Hence the percent fatty acid calculated as shown in the tables may not be accurate weight percent of fatty acids present.

Fatty acids were identified by comparing retention times with those of authentic standards and by GLC-Mass Spectrometry. GLC-MS provided only molecular weight of the individual component. This identification method does not rule out the possibility that a non fatty acid component having molecular weight and retention time similar to C22:0, C24:0, and C26:0 fatty acids is present instead of long chain fatty acids.